Immuno-fluorescence study of five zygomycetous fungi with two chitin-binding probes

by

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A Thesis
submitted to the department of Biological Sciences
in partial fulfillment of the requirements
for the degree of
Master of Science

September, 1993
Brock University
St. Catharines, Ontario

Abstract

A polyclonal antiserum was prepared against a purified microsomal chitinase isolated from the fungus *Choanephora cucurbitarum.* Indirect immuno-fluorescence was used to localize chitinase at various developmental stages of five zygomycetous fungi and during a biotrophic mycoparasite interaction with a susceptible and resistant host. This was compared to localization of oligomers of N-acetylglucosamine with the lectin wheat germ agglutinin (WGA). Dot-immunoblot and Western blot techniques revealed that the anti-serum reacted strongly with the antigen from which it was derived. Cross reactivity of the antiserum was found with WGA and another chitin binding lectin, *Phytolacca americana* agglutinin (PAA). Immuno-fluorescence results showed the direct involvement of chitinase in spore swelling, germination, sporangium development and response during mechanical injury. There appeared to be no involvement of chitinase during apical hyphal growth or new branch initiation in any of the fungi tested despite mild proteolysis and permeabilization of the cell surface prior to labelling. Binding with WGA revealed similar patterns of fluorescence to that of chitinase localization but differed by showing fluorescence and therefore chitin localization at the apex and new branch initiation when tested at different developmental stages. There was no difference between chitinase localization and binding with WGA in a susceptible host and resistant host challenged with the mycoparasite, *Piptocephalis virginiana.* Differences in binding ability of antichitinase and lectin WGA suggests that the latter is not a suitable indicator for indirect localization of the lytic enzyme, chitinase.

Acknowledgements

I wish to express my sincere gratitude to my supervisor, Professor M. S. Manocha for his unending patience and tolerance with my work and without whom this thesis would never have been completed. I also appreciate the help of Dr. A. Castle and Dr. R. Balasubramanian for their valuable advice and many discussions throughout the entire period of my stay at Brock University. Dr. Balasubramanian raised the polyclonal antiserum against the purified chitinase used in this study.

I dedicate this thesis to my family who provided love, moral support and encouragement to pursue further studies.

Many thanks are also due to Janet Pinder, the rest of the department and support staff for their assistance and Mike Lozon for his help with the figures.

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Introduction

Chitinase hydrolyzes chitin, the unbranched homopolymer of N-acetylglucosamine (GlcNAc) in a ß-1,4 linkage. Chitin is a structural component in the cell walls of fungi and of some algae; in the shells or cuticles of arthropods including crustaceans and insects; in nematodes; in mollusks; in worms and in many other types of organisms (Cabib, 1987; Godday, 1990).

Chitin microfibrils are a major structural component of the cell wall of most filamentous fungi. In the yeast *Saccharomyces cerevisiae*, short chitin micrifibrils are located almost exclusively in the primary septum, of which it seems to be the sole component (Cabib, 1987). Its presence together with that of other polysaccharides has been used as a criterion for fungal taxonomy (Bartnicki-Garcia, 1968). In fungi, chitinase is a morphogenetic enzyme which plays an integral part in fungal cell wall metabolism.

Cell wall growth, including growth at the apex in filamentous fungi, has been proposed to be the result of a harmonious balance between the processes of synthesis and lysis by enzymes such as chitin synthetase (EC 2.4.1.16) and chitinase, respectively (Bartnicki-Garcia, 1973). Essentially, lytic enzymes permanently loosen the newly deposited rigid cell wall at the apex which creates gaps for the insertion of new cell wall material, or growth by intussusception. However, it has been argued that evidence for participation of lytic enzymes such as chitinase in cell wall growth, particularly in apical growth, is indirect and circumstantial (Wessels, 1986). Instead, it has been suggested that the cell wall material deposited at the growing apex by apposition of new cell wall polymers is inherently plastic rather than rigid but gradually develops stiffness due to secondary processes occurring in the wall (Wessels, 1986). Others have investigated gradients of membrane associated Ca^{2+} (Yuan and Heath, 1991), actin (Heath, 1990; Roberson, 1992) and the role of turgor pressure (Kaminskyj et al., 1992) as associated with apical growth.

Despite the apparent controversy, lytic enzymes such as chitinase may be involved in the net degradation of wall polymers during starvation, degradation of septa for nuclear migration, fusion of hyphae such as during anastomosis and clamp connections, branch initiation, spore swelling, germination, autolysis, gross autolysis associated with stipe elongation and spore dispersal in *Coprinus lagopus,* budding in *Candida albicans*, and cell separation in *S. cerevisiae* (Iten and Matile, 1970; Bartnicki-Garcia, 1973;

Vessey and Pegg, 1973; Elango et al., 1982; Isaac and Gokhale, 1982; Barrett-Bee and Hamilton, 1984; Wessels, 1986; Pedraza-Reyes and Lopez-Romero, 1989; Reyes et al., 1989; Kuranda and Robbins, 1991; Cabib et al., 1992).

There is a distinct possibility that both chitinase and chitin synthetase play roles in the early events of host-parasite interaction between susceptible and resistant Mucoraceous hosts to the biotrophic haustorial mycoparasite *Piptocephalis virginiana* (Manocha, 1987). Others support the contention that chitinase may be involved in similar events between entomopathogenic fungi, necrotrophic mycoparasites and vesicular arbuscular mycorrhizal (VAM) fungi and their hosts (Ordentlich et al., 1991; St. Leger et al., 1991; Dumas-Gaudot et al., 1992; Lambais and Mehdy, 1993). Excellent introductions to chitinases in general can be found in the reviews by Stirling et al., (1979) and Cabib (1987).

It is clear that the presumptive role of chitinase in the aforementioned morphogenetic events of filamentous fungi has yet to be elucidated and may be due to lack of techniques for spatial localization of chitinase in the fungal cell. Localization of oligomers of GlcNAc with the lectin wheat germ agglutinin (WGA) during a necrotrophic mycoparasite-fungal host interaction has been used as an indirect probe for chitinase due to the localization of the degradation products of this enzyme (Cherif and Benhamou, 1990).

A polyclonal antiserum was prepared against a purified microsomal chitinase isolated from the mucoraceous fungus *Choanephora cucurbitarum* (Balasubramanian and Manocha, 1992). Specificity of the antiserum was confirmed by dot-immunoblot and Western blot techniques. Indirect immuno-fluorescence technique was employed to localize chitinase during various developmental stages in the life cycle of *C. cucurbitaurm* and four other related fungi. This was compared to the localization of oligomers of GlcNAc with WGA as an indirect probe for chitin through a direct lectin binding assay. Both probes were also employed during early stages of parasitism in the susceptible host, *C. cucurbitaurm* and the resistant host, *Phascolomyces articulosus* challenged with the biotrophic haustorial mycoparasite *Piptocephalis virginiana*. Comparison of chitinase localization with that of oligomers of GlcNAc which comprise chitin was performed to test the suitability of using a lectin (WGA) as an indirect probe for the enzyme, chitinase.

Literature Review

Mode of Chitin Degradation

The nomenclature of chitinolytic enzymes is still marred with confusion. There is sufficient evidence to divide chitinase activity into two principal types: endochitinases and exochitinases (Robbins et al., 1988; Bade and Hickey, 1989; Rast et al., 1991). The final products formed by endochitinases (EC 3.2.1.14) are soluble, low molecular weight multimers of GlcNAc such as chitotetraose and chitotriose with the dimer, di-acetylchitobiose, being predominant. Cleavage occurs randomly at internal points over the entire length of the chitin microfibril. On the other hand, exochitinases (EC 3.2.1.14) catalyze the progressive release of di-acetylchitobiose in a stepwise fashion and no monosaccharides or oligosaccharides are formed (Fig. 1). Cleavage occurs only at the nonreducing end of the chitin microfibril. This enzyme is refered to as chitobiosidase in a recent study (Harman et al., 1993).

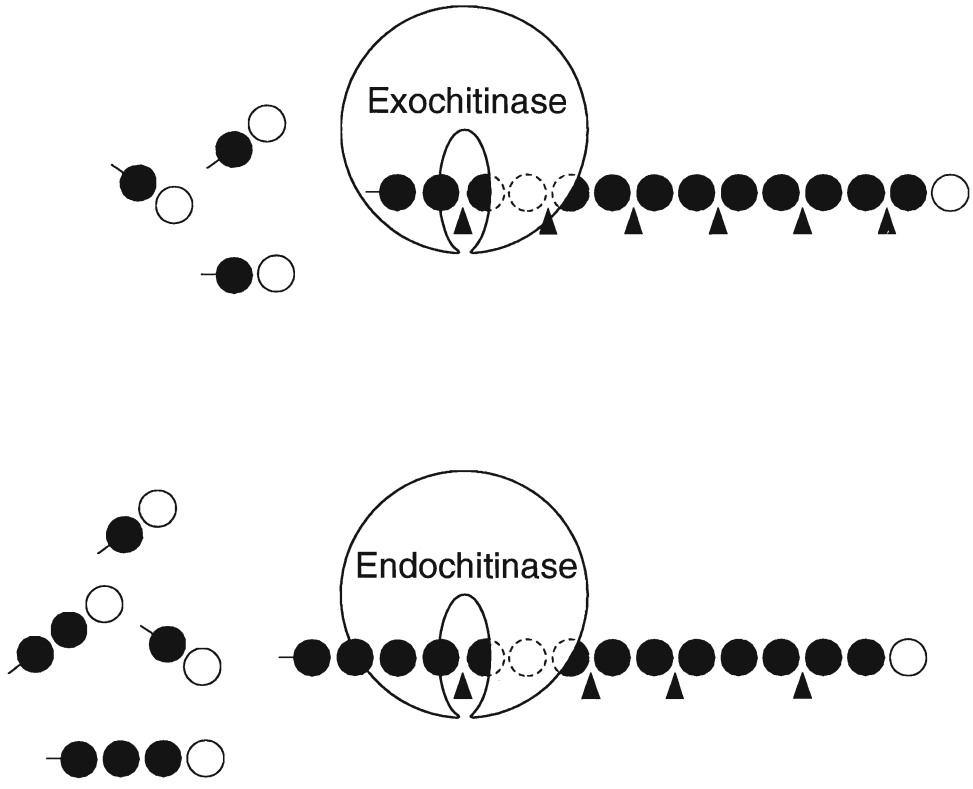
ß-1,4 N-acetylglucosaminidase (EC 3.2.1.30) splits di-acetylchitobiose as well as chitotriose and chitotetraose into GlcNAc monomers in an exo type fashion. This definition inherently includes the so called chitobiase activity for hydrolysis of di-acetylchitobiose, but has long been classified under ß-1,4 N-acetylglucosaminidase. Hydrolysis of terminal nonreducing N-acetylhexosamine residues in N-acetylhexosaminides such as N-acetylgalactosaminides is carried out by N-acetylhexosaminidase (EC 3.2.1.52).

Fungal Chitinases

Nature, Subcellular Localization and General Properties

Fungal chitinases have been localized extracellularly, in the periplasmic space and the plasma membrane. Evidence for the membrane bound zymogenic nature of fungal chitinases stemmed from the inability to isolate purified microsomal chitin synthetase preparations freely capable of producing nascent chitin (McMurrough and Bartnicki-Garcia, 1973; Molano et al., 1979). Often, these microsomal preparations yielded di-acetylchitobiose instead of chitin upon incubation with UDP-N-acetylglucosamine which is the substrate

Fig. 1. Original model comparing the mode of chitin degradation by exochitinase and endochitinase. See text for explanations.


Exochitinase
Endochitinase
= GlcNAc
= Chitin microfibril
= Nonreducing terminal
= Reducing terminal
= Site of hydrolysis

1

for chitin synthetase. Subsequently, a chitinase was found that degrades nascent chitin as fast as it was made by chitin synthetase (Molano et al., 1979; Lopez-Romero et al., 1982; Humphreys and Gooday, 1984). Chitinase activity was stimulated by treatment of microsomes with commercial proteases such as trypsin and inactivation occurred after treatment with commercial phospholipases. This suggested the requirement for phospholipid-enzyme interaction for activity; a similar result found with chitinases from a diverse source of fungi: *Candida albicans* (Dickinson et al., 1991); *Choanephora cucurbitarum* (Manocha and Balasubramanian 1988); *Mucor mucedo* (Humphreys and Gooday 1984); *M. rouxii* (McMurrough and Bartnicki-Garcia, 1973); *Neurospora crassa* (Zarain-Herzberg and Arroyo-Bergovich 1983) and *Piptocephalis virginiana* (Balasubramanian and Manocha 1986).

Fungal chitinase activity is found in different subcellular fractions beside microsomal preparations. In *N. crassa,* chitinase activity occurs in membrane rich fractions, but most of the activity is distributed either in the cell wall or cytosolic fractions, approximately 30% and 50%, respectively (Zarain-Herzberg and Arroyo-Bergovich 1983). Chitinases also occur in the cell wall of *Aspergillus nidulans* (Polacheck and Rosenberger 1978); in cytosolic and membrane fractions of *C. cucurbitarum* and *P. articulosus* (Balasubramanian and Manocha, 1992); in mixed membrane, cytosolic and cell wall fractions of *M. rouxii* (Pedraza-Reyes and Lopez-Romero, 1989) and in the periplasm, vacuoles (Elango et al., 1982) and the cell wall (Kuranda and Robbins, 1991) of *S. cerevisiae.* Cell free extracts of both mycelial (McMurrough and Bartnicki-Garcia, 1973) and yeast phase cells (Lopez-Romero et al., 1978) of the dimorphic fungus *M. rouxii* have revealed chitinases which interfere with chitin biosynthesis *in vitro.*

Some general properties of fungal chitinases are present in Table 1. It can be seen that they are active at slightly acidic pH levels; have high apparent temperature optima; have a high degree of stability which may be due to glycosylation; endochitinase and some exochitinase activity is present; the molecular masses differ considerably with higher molecular masses present in *C. albicans, S. cerevisiae* and *Verticillium albo-atrum* (Correa et al., 1982; Pegg, 1988; Dickinson et al., 1989). They are inhibited by copper and mercury salts, lack ionic cofactors and are competitively inhibited by allosamidin. Exponentially growing mycelia of *M. rouxii* have certain Type B soluble endochitinases which unlike other fungal chitinases are basic (or neutral),

Table 1
General properties of various fungal chitinases

Organism	pH optimum	Temperature optimum	Molecular mass (kDa)	Inhibitory agents	Mode of chitin degradation	Reference
Aphanocladium album Chitinase 1						
(extracellular)	4.0	50°C*	39[a], 20[b]	Fe^{2+}, Hg^{2+}	Endo	[1]
Aspergillus nidulans						
(extracellular)	5.0	-	27	Hg^{2+}, Cu^{2+}, Ca^{2+}, Ag^{2+}	Endo	[2]
Candida albicans						
(cytosolic)	6.5	45°C*	70	allosamidin	Endo	[3,4,5]
(microsomal)	8	45°C*	-	allosamidin	Endo	
Choanephora cucurbitarum						
(cytosolic)	6.0	40°C*	55 & 69.5[a]	Cu^{2+}, Hg^{2+}, Mn^{2+}, KCN	Endo	[6]
(microsomal)	4.5-6.0	40°C*	66	Cu^{2+}, Hg^{2+}, Mn^{2+}	Endo	
Gliocladium virens (strain 41)						
(extracellular)	4.0-6.0	-	41	-	Endo	[7]
Metarhizium anisopliae						
(extracellular)	5.3	38-45°C*	33 & 66[b]	-	Exo	[8]
Mucor mucedo						
(cytosolic)	5.7	-	-	Cu^{2+}	Endo	[9]
(microsomal)	5.6	-	-	Cu^{2+}, PMSF	Endo	
Mucor rouxii						
(cytosolic, A	-	-	53	-	Exo	[10]
4 h culture) B	-	-	28	-	Exo	
(cytosolic, CI	6.5	30°C	30.7	-	Exo	
10 h culture) CII	6.5	30°C	24.2	-	Exo	
Neurospora crassa						
(cytosolic)	6.7	-	20.6	Cu^{2+}, Hg^{2+}	Endo	[11]
Phascolomyces articulosus						
(cytosolic)	-	40°C*	53 & 69.5[a]	Cu^{2+}, Hg^{2+}, Mn^{2+}, KCN	Endo	[6]
(microsomal)	-	40°C*	66	Hg^{2+}, Mn^{2+}, EDTA	Endo	
Saccharomyces cerevisiae						
(cytosolic)	2.5	-	130	-	Endo	[12,13]

Trichoderma harzianum (strain P1)						
(extracellular)	4.0-7.0	-	35, 40	-	Exo	[14]
(extracellular)	4.0	-	41	-	Endo	
Trichoderma harzianum (strain 39.1)						
(extracellular)	4.5	40°C	40[a], 36[b]	Zn^{2+}	Endo	[15]
Verticillium albo-atrum						
(cytosolic)	5.1	45°C*	64	Cu^{2+}, Hg^{2+}, KCN	Endo	[16,17]

- not tested, * stable at low temperature, [a] by SDS-PAGE, [b] by gel filtration, EDTA=ethylenediaminetetraacetic acid, KCN=potassium cyanide PMSF=phenylmethylsulfonyl fluoride

1=Kunz et al., (1992) 2=Reyes et al., (1989) 3=Barrett-Bee and Hamilton, (1984) 4=Dickinson et al., (1989) 5=Dickinson et al., (1991) 6=Balasubramanian and Manocha, (1992) 7=Di Pietro et al., (1993) 8=St. Leger et al., (1991) 9=Humphreys and Gooday, (1984) 10=Pedraza-Reyes and Lopez-Romero, (1989) 11=Zarain-Herzbaerg and Arroyo-Begovich, (1983) 12=Correa et al., (1982) 13=Kuranda and Robbins, (1991) 14=Lorito et al., (1993) 15= Ulhoa and Peberdy, (1992) 16=Vessey and Pegg, (1973) 17=Pegg, (1988)

nonzymogenic and degrade preformed chitin (Rast et al., 1991). Competitive inhibition of all chitinases was found with chitobionolactone oxime (Rast et al., 1991).

Role in Morphogenesis

Bartnicki-Garcia (1973) was the first to propose that fungal cell wall morphogenesis is the result of a delicate balance between synthesis by chitin synthetase and lysis by chitinase. There are a number of examples which support this proposal. In several cases, maximal chitinase activity occurs in conjunction with maximal chitin synthetase activity during growth, indicating that the former is also critical for growth. For example, high speed supernatant fractions of the yeast form of *C. albicans* at the rapid budding phase of growth showed a peak of chitinase activity which paralleled that of chitin synthetase activity (Barrett-Bee and Hamilton, 1984). Microsomal preparations of chitinase in *C. albicans* also had maximal activity occurring together with chitin synthetase (Dickinson et al., 1991). High chitinase activity has been correlated with the onset of germination (4 h cultures) in cytosolic extracts of mycelial *M. rouxii* and at mid-exponential phase of growth (10 h cultures) (Pedraza-Reyes and Lopez-Romero, 1989); the latter peak paralleled that of chitin synthetase activity. Analysis of the 10 h peak revealed the presence of two antigenically distinct, exochitinases with different molecular masses and ionic charges that degraded nascent chitin and yielded chitobiose as the sole product. These chitinases were proposed to be involved in hyphal extension and branching. The same group also described the identification of nine chitinase species associated with the 4 h peak expressed in swollen germ spheres just prior to the appearance of the germ tube (Pedraza-Reyes and Lopez-Romero, 1991). Cabib et al. (1992) confirmed chitinase lysis of the chitin ladened septum during the process of cell separation in *S. cerevisiae.* Chitin synthetase was shown to act as a repair enzyme by replenishing chitin during cytokinesis.

Rast et al. (1991), being critical that *M. rouxii* may not have consisted entirely of hyphae during full exponential growth in the experiments of Pedraza-Reyes and Lopez-Romero (1989), further investigated chitinolytic activity in the same fungus. Exponentially growing short-branched hyphae contained at least seven chitinases capable of random cleavage of ß-1,4 bonds, suggestive of endochitinases. Soluble chitinases were divided into two classes, Type A and

B. Type A chitinases were acidic, displayed partial latency, showed an unusual affinity to dextran gel and acted weakly on colloidal chitin. Type B chitinases were basic (or neutral) and not zymogenic, did not behave anomolously upon gel filtration and could degrade preformed chitin. Distinct ß-1,4 N-acetylglucosaminidase activity was concomitantly produced with chitinase activity. It was proposed that chitinase and ß-1,4 N-acetylglucosaminidase act synergistically to degrade the native substrate in growing hyphae. Moreover, a new hypothetical model of a triple integrated enzyme system consisting of chitinase, ß-1,4 N-acetylglucosaminidase and chitin synthetase was suggested for controlled chitin metabolism at sites of hyphal branch initiation (Rast et al., 1991).

Early studies by Coudron et al. (1984) demonstrated that chitinolytic activity in several entomopathogens was important for growth and potentially needed for penetration. Chitinase activity compared to the rate of fungal development in isolates of *Nomuraea rileyi,* which is parasitic on larvae of *Trichoplusia ni*, the cabbage looper, showed significantly higher levels of an endochitinase and ß-1,4 N-acetylglucosaminidase in two virulent *N. rileyi* strains compared to an avirulent mutant strain grown over a period of 30 days (El-Sayed et al., 1989). In the virulent isolates, the chitinase activity/total protein ratio during germination (2 days) was as much as 35 times greater than that found in conidia at day 0. Chitinase activity was also present at the onset of the blastospore stage (3 days) which is a stage critical to penetration of the chitin ladened host insect cuticle. Thus, it was speculated that chitinolytic enzymes play a role in dissolution of insect cuticles during penetration by entomopathogenic fungi.

The correlation of chitinase and other glycosidases with autolysis has been well examined in the infection of tomato by the vascular wilt pathogen *Verticillium albo-atrum.* An endocellular chitinase produced by *V. albo-atrum* hyphae and culture filtrates released GlcNAc from pure chitin and autoclaved *V. albo-atrum* hyphae (Vessey and Pegg, 1973). Rapid loss of mycelium by autolysis between 3 and 6 days of growth was demonstrated in shake and stir culture experiments. Chitinase activity was suggested to be proportional to the loss of mycelium, i.e. rate of autolysis, a concept supported with further fungal studies: *Aspergillus nidulans* (Isaac and Gokhale, 1982; Reyes et al., 1989) and *Fusarium solani*, *Glomerella cingulata* and *Helminthosporium victoriae* grown on membranes under a low nutrient regime (Ko and Lockwood, 1970).

Subsequently, the finding of a ß-1,3 glucanase (EC 3.2.1.39) and a ß-glucosidase (EC 3.2.1.21), active on ß-1,3 glucans, in the culture filtrate of *V. albo-atrum* supported a prior notion that autolysis was associated with more than one enzyme (Young and Pegg, 1981).

Iten and Matile (1970) correlated gross autolysis associated with stipe elongation and spore dispersal in *Coprinus lagopus* with the action of an endocellular chitinase synthesized shortly after autolysis began in the gills. A second group of hydrolytic enzymes: acid and alkaline proteases, RNase, phosphatase and ß-glucosidases were found in the vacuoles isolated from vegetative mycelium as well as gills and therefore these enzymes were postulated to have both an intracellular and extracellular role.

Elango et al. (1982) first described an endochitinase in *S. cerevisiae* which was found to be a secretory protein because of detection of chitinase activity in a medium containing isolated protoplasts. It was proposed that this chitinase was localized in the periplasmic space and was thus, strategically positioned to attack chitin secreted into that space during septum formation. This chitinase also was speculated to play a role in the final fission of septa leading to cell separation (Elango et al., 1982). Using colloidal gold-labelled anti-*Serratia marcescens* chitinase antibody, Harris and Szaniszlo (1986) showed localization along the inner portion of the cell wall and at the scar region in the human pathogenic yeast *Wangiella dermatidis.* Disruption of the *S. cerevisiae* chitinase gene did indeed lead to a defect in cell separation (Kuranda and Robbins, 1991), which supported the initial speculation by Elango et al. (1982), and was confirmed by Cabib et al. (1992).

Host-Parasite Interaction

Whether chitinase is involved in penetration of host insect cuticle by entomopathogenic fungi is far from clear. Endochitinase, ß-1,4 N-acetylglucosaminidase and other hydrolytic enzyme activities are present when three entomopathogens: *Beauveria bassiana*, *Metarhizium anisopliae,* and *Verticillium lecanii* are grown on locust cuticles as the sole carbon source (St. Leger et al., 1986). Five strains of *M. anisopliae* obtained from widely divergent sources for the production of degradative enzymes were grown on defined media and on purified cuticles of *Galleria mellonella,* the greater wax moth and *Trichoplusia ni.* These strains yielded highest levels of proteases and

endochitinases from cuticle grown cultures (Gupta et al., 1991).

However there are examples of studies which do not support chitinase involvement in penetration. *Metarhizium anisopliae* does not appear to produce chitinase during penetration of cuticle of *Manduca sexta* (St. Leger et al., 1987). Similarly, a chitinase deficient mutant of *V. lecanii* was just as pathogenic to aphids as the wild type (Jackson et al., 1985). Conclusive proof for the crucial involvement of chitinolytic (or other) enzymes during penetration would require that the enzyme be present at the site of penetration, enzyme deficient mutants must have altered virulence, the transfer of the enzyme gene into these mutants should restore pathogenicity and specific inhibitors should block the penetration step and protect insects from disease (St. Leger et al., 1991). To meet all these points, extensive *in vitro* biochemical characterization must be made, as in the case of *M. anisopliae* (St. Leger et al., 1991). It could be also easily conceived that different entomopathogen-host insect interactions may differ in their penetration mechanisms.

Chitinases have been implicated to play a role in penetration of fungal hosts of the biotrophic mycoparasite *Piptocephalis virginana* (Manocha, 1987). Penetration by the infection hypha is thought to occur by both mechanical pressure and enzymatic degradation. Ultrastructural studies often show an inpushing of the host cell wall and erosion of the outer layer by the advancing infection hyphae (Manocha and Golesorkhi, 1981). Only negligible levels of chitinase and chitosanase activity were found in culture filtrates of *P. virginiana* suggesting strict regulatory control indicative of the biotrophic nature of the mycoparasite and judicious deployment of the enzyme (Balasubramanian and Manocha, 1986).

Chitinases and related lytic enzymes may be involved in colonization of host plants by vesicular arbuscular mycorrhizal (VAM) fungi; order Endogonales, class Zygomycetes (Spanu et al., 1989; Dumas-Gaudot et al., 1992). Even though VAM fungi are obligate symbionts, they trigger peroxidase and chitinase induction in mycorrhizal roots of *Allium porrum* (leek) as in the case for pathogenic microorganisms (Spanu et al., 1989). Dumas-Gaudot et al. (1992) showed that chitinase activities were always higher in roots of leek, onion and pea colonized by various *Glomus* sp. compared to the non-mycorrhizal plants. Several additional isoforms of chitinase were induced over constitutive chitinases present in control roots. Enhancement of chitosanase (EC 3.2.1.99) activity was also found in leek and onion roots while there was a

lack of any ß-1,3 glucanase activity in colonized leek and onion roots. This correlates with the fact that VAM fungi, together with other Zygomycetous fungi, contain mainly chitin and chitosan in their cell walls and lack ß-1,3 glucans (Bartnicki-Garcia, 1968), but the increased chitinase could be of plant origin.

Endochitinase and ß-1,3 glucanase gene expression was recently studied in bean roots infected with *Glomus intraradices* in order to see test if a correlation existed between increased enzyme expression with inhibition of intraradial fungal growth seen under high phosphorous (P) conditions (Lambais and Mehdy, 1993). Expression of both enzymes was suppressed to a greater extent at low P concentrations than at high P concentrations. In contrast to above, expression was high during the early stages of fungal colonization followed by a suppression of activity at later stages. Generally, the results suggested suppression of plant defense mechanisms at low and high P concentrations and during the latter, lower levels of endochitinase suppression may contribute to reduced root colonization (Lambais and Mehdy, 1993).

Mycoparasitism by several strains of *Trichoderma* spp. has been correlated with the presence of lytic enzymes such chitinase and ß-1,3 glucanase. Both enzymes are induced by different strains of *Trichoderma harzianum* in culture media supplied with cell walls of *Rhizoctonia solani* and *Sclerotium rolfsii* as the sole carbon source (Elad et al., 1982). Direct degradation of purified cell walls of *Botrytis cinerea* has also been shown by *T. harzianum* (strain CECT 2413) (De La Cruz et al., 1992). Inhibition of spore germination and germ tube elongation of nine chitin containing test fungi was caused by endochitinase and exochitinase activity from *T. harzianum* strain P1 (Lorito et al., 1993). A synergistic increase of antifungal activities resulted when the two enzymes were combined (Lorito et al., 1993). Another fungal endochitinase, purified from *Gliocladium virens* strain 41 showed antifungal activity against *B. cinerea*, although with lower efficacy (Di Pietro et al., 1993).

Lytic activity in *Trichoderma-Rhizoctonia* and *Trichoderma-Sclerotium* has been correlated with the degree of biological control *in vivo* (Elad et al., 1982; Sivan and Chet 1989). Such is not the case in interactions of *T. harzianum* strains mycoparasitic against *Fusarium oxysporum* f. sp. *melonis* and *F. o.* f. sp. *vasinfectum,* parasitic on muskmelon and cotton, respectively. Sivan and Chet (1989) demonstrated that ß-1,3 glucanase and chitinase were released by *T. harzianum*, but two strains of *T. harzianum* failed to colonize both host species *in vitro*. Production of extracellular chitinase by a *Trichoderma* sp.

mycoparasitic on *Fusarium oxysporum* f. sp. *radicis-lycopersici* was shown by Cherif and Benhamou (1990) using colloidal-gold immunocytochemical technique. Oligomers of GlcNAc were released from *F. o.* f. sp. *radicis-lycopersici* into the growth medium, starting 48 h after inoculation and at subsequent times thereafter. Colloidal gold-labelled Wheat Germ Agglutinin (WGA) served as an indirect probe for chitinase localization. Three to five chitinolytic bands on sodium dodecyl sulfate (SDS) gels were resolved from cultures containing host cell wall fragments (Cherif and Benhamou,1990). Sivan and Chet (1989) speculated that the cell walls of these *Fusaria* spp. are apparently more resistant to lysis than other fungal cell walls because of an outer layer of protein present on *Fusaria* cell walls. Other mechanisms may be involved in the biological control activity of *Trichoderma-Fusaria* interactions (Ordentlich et al., 1991). Biochemical characterizations of chitinase 1 isolated from the culture filtrate of the hyperparasite *Aphanocladium album* suspected to be involved in host fungal cell wall degradation has been reported (Srivastava et al., 1985; Kunz et al., 1992).

Regulation of Chitinase Gene Expression

Chitinase gene expression in fungi and other microorganisms is thought to be controlled by a repressor-inducer system in which chitin or the products of degradation (oligomers) serve as inducers. St. Leger et al. (1986) investigated the mode of regulation of chitinolytic enzymes produced by *Metarhizium anisopilae* which is the causative agent of green muscardine disease in pecan weevils. Their work revealed that chitinase and ß-1,4 N-acetylglucosaminidase activities occurred after the appearance of proteolytic enzymes when the pathogen was grown in the presence of host cuticle. Either chitinase was induced as chitin became available after degradation of encircling cuticular proteins or chitinase was released from the cuticle following its solubilization. Chitinase synthesis was proposed to be regulated by the products of chitin degradation through an inducer-repressor mechanism. High levels of chitinase were found only in cultures supplied with chitin, but not pectin, xylan or cellulose; the most effective inducer was GlcNAc. Glucosamine was capable of inducing chitinase since chitin from natural sources appears to be partially deacetylated while chitosan contains approximately 10 to 20% acetylated residues (St. Leger et al., 1986). Induction of chitinase expression by GlcNAc

and repression by glucose also occurs in a chitinase overproducing mutant of *A. album* (strain E3) (Vasseur et al., 1990) and in the entomopathogen *Beauveria bassiana* (Smith and Grula, 1983). Blaiseau et al. (1992) examined regulation of chitinase at the level of gene expression in *A. album* E3. Northern blot analysis revealed induction of chitinase 1 mRNA of *A. album* E3 grown in the presence of chitin and lack of gene expression when grown in glucose.

Ulhoa and Peberdy (1991; 1992; 1993) addressed chitinase regulation as well as biochemical characterization in *Trichoderma harzianum* 39.1. High chitinase activity was found only in cultures supplied with chitin but not with cellulose, chitosan or chitobiose. Contrary to that found in the above three cases, GlcNAc did not promote enzyme production in *T. harzianum* 39.1; GlcNAc and glucose repressed chitinase synthesis (Ulhoa and Peberdy, 1992). The same was true of ß-1,4 N-acetylglucosaminidase activity (Ulhoa and Peberdy, 1993).

Together, these studies demonstrate that formation of chitinase in fungi is controlled by an inducer-repressor mechanism in which chitin or products of degradation (oligomers) serve as an inducer. Constitutive levels of chitinase activity was demonstrated in *A. album* E3 and *T. harzianum* 39.1 (Vasseur et al., 1990; Ulhoa and Peberdy, 1991). Trace quantities of constitutive enzymes continuously released, even under starved conditions, may be sufficient to initiate chitin degradation and release soluble oligomers.

Repression by glucose in *A. album, B. bassiana* and *M. anisopilae,* and by glucose and GlcNAc in *T. harzianum* 39.1 suggests that catabolite repression or the so called glucose effect may be involved in the regulation of chitinase synthesis (Vasseur et al., 1990; Smith and Grula, 1983; Ulhoa and Peberdy, 1991). Both chitinase and chitosanase can be subject to catabolite repression in *M. anisopilae* (St. Leger et al., 1986). Clearly, fungal chitinases, like other enzymes supplied with exogenous substrates, are inducible and subject to catabolite repression.

Molecular Biology and Chitinase Gene Cloning

The gene (CTS-1) for a *Saccharomyces cerevisiae* endochitinase has been sequenced following initial cloning by a plasmid based overexpression (Kuranda and Robbins, 1991). Analysis of the derived amino acid sequence suggests that the protein contains four domains: a signal sequence, a catalytic

domain, a serine/threonine rich region and a chitin binding domain at the carboxy terminal (Fig. 2). Interestingly, the catalytic domain was found to be clearly homologous to a pathogenesis related cucumber chitinase. Two small regions of this domain were conserved with the cucumber chitinase, several bacterial chitinases, endoglycosidase H, and a mammalian lysozymal chitinase; all of which cleave the ß-1,4 glycosidic bond between adjacent GlcNAc residues (Kuranda and Robbins, 1991). Using a semipurified chitinase from yeast cells of *C. albicans,* chemical modification studies with the use of group specific reagents suggested the presence of Glu/Asp carboxyl group(s) at or near the active site, that were important for enzyme activity (Milewski et al., 1992).

Description of the chitin binding domain in chitinase from*S. cerevisiae* is noteworthy because a number of cellulose and starch degrading enzymes incorporate extended high affinity binding domains for their respective substrates (Knowles et al., 1987). The binding domain is required for localization of the enzyme to the yeast cell wall and this enzyme is required for cell separation (Kuranda and Robbins, 1991; Cabib et al., 1992).

Isolation and sequencing of genes encoding for chitinase I and chitinase II has been reported in *Rhizopus oligosporus* (Yanai et al., 1992). In comparison to *S. cerevisiae* chitinase, the structures of the products of the *chi1* and *chi2* genes have five domains instead of four, with an additional C-terminal domain present (Fig. 3). Two small regions of the catalytic domain were also found to be conserved with the ß-1,4 glycosidases mentioned above. Comparison of the deduced amino acid sequences from the nucleotides with the C-terminal amino acid sequences of the purified chitinases indicated processing of the precursors at the C termini. It was suggested that this domain contains two regions: (i) a variable region whose sequence is different for the two chitinases and where the C terminal processing event occurs and (ii) a prosequence region which is removed during maturation of these enzymes (Yanai et al., 1992). The structures of these chitinases are very similar to the other fungal carbohydrolases, but the existence of the C-terminal domain is specific for the chitinases (Knowles et al., 1987).

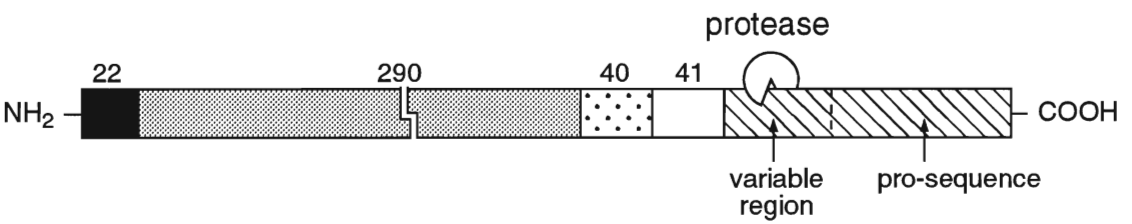
The first filamentous fungal chitinase gene sequence was obtained from *Aphanocladium album* (Blaiseau et al., 1992). An antiserum produced against chitinase 1 from a chitinase overproducing mutant of *A. album* E3 was used to select a suitable cDNA clone using standard techniques. Heterologous

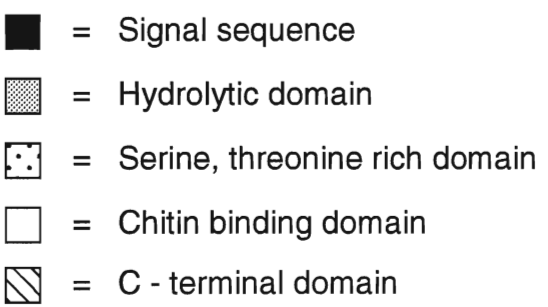
Fig. 2. Model for primary preprotein structure of chitinase of *Saccharomyces cerevisiae* derived from cDNA clones. Numbers above refer to the number of amino acids in each domain. The signal sequence is cleaved and the serine, threonine rich domain is glycosylated with sugar chains containing 2-5 mannose residues post-translationally to yield the mature protein. Redrawn with permission from Kuranda and Robbins (1991).

Fig. 3. Model for primary preprotein structure of chitinase of *Rhizopus oligosporus* derived from cDNA clones. Note the prescence of an additional domain comprised of two regions at the C-terminal. Redrawn with permission from Yanai et al. (1992).


20
306
52
81
NH2
COOH

2


protease
22
290
40
41
NH2
COOH
variable region
pro-sequence


= Signal sequence
= Hydrolytic domain
= Serine, threonine rich domain
= Chitin binding domain
= C - terminal domain

3

transformation of the putative chitinase genomic fragment to two strains of *Fusarium oxysporum* f. sp. *melonis* resulted in high chitinase activity in the culture filtrates over the control and did not inhibit growth. Only a partial amino terminal, 23 amino acid sequence was obtained by direct sequencing of the chitinase 1 gene. Therefore, no comparison can be made to known complete amino acid sequences of chitinases from *S. cerevisiae* or *R. oligosporsus.*

Role of Plant Chitinases and Glucanases in Anti-Fungal Defense

Although this thesis deals with fungal chitinases, properties of plant chitinases must be mentioned because of the possible involvement as an active defense mechanism against chitinous plant pathogens such as fungi or insects. Higher plant cell wall polysaccharide microfibrils are composed of cellulose, a polymer of glucose in a ß-1,4 linkage, not chitin. Chitin does not exist in plant cells and in no case has an endogenous plant substrate been found for purified chitinase (Boller, 1987). Even so, chitinases have been purified from a large number of plants encompassing diverse taxonomic groups. In fact, several recent studies have proposed a morphogenetic role for plant chitinase (Harikrishna et al., 1991; Kragh et al., 1991; Samac and Shah, 1991; del Campillo et al., 1992; de Jong et al., 1992; Leung, 1992; Swegle et al., 1992).

General biochemical properties of many plant chitinases has been compiled by Bol et al. (1990). In comparison to fungal chitinases, plant chitinases are generally endochitinases; have molecular masses in the range of approximately 30 kDa and are acidic or basic in nature (the acidic forms are secreted into the apoplast or extracellular environment while the basic forms accumulate intracellularly in the vacuole).

Perhaps more than any other aspect of the research on plant chitinases has been the observation that they are anti-fungal in nature. It has been implicitly stated that the *in vivo* role of these 'pathogenesis related' (PR) proteins is to protect the host from invasion by fungal pathogens, and that as such, they are an integral part of a general disease-resistance mechanism (Verburg and Huynh, 1991; Huynh et al., 1992). Purified plant chitinases and ß-1,3 glucanases attack and partially digest isolated cell walls of potentially pathogenic fungi (Young and Pegg, 1982; Bol et al., 1990) and are potent inhibitors of fungal growth (Boller et al., 1983; Schulbaum et al., 1986; Roberts and Selitrennikoff, 1988; Jacobsen et al., 1990; Verburg and Huynh, 1991;

Dumas-Gaudot et al., 1992; Huynh et al., 1992;). Moreover, fungal elicitors which induce other defense related responses such as phytoalexin synthesis, can induce chitinase as well as ß-1,3 glucanase in several plants (Abeles et al., 1971; Pegg and Young, 1981; Keen et al., 1983; Mauch et al., 1984; Roby et al., 1986; Kurosaki et al., 1987; Hedrick et al., 1988; Kombrink et al., 1988).

Both chitinase and ß-1,3 glucanase are coordinately induced during attack by fungal pathogens (Mauch et al., 1984; Pegg and Young, 1981; Mauch et al., 1988a) and by fungal elicitors (Mauch et al., 1984; Kombrink et al., 1988). Chitinase and ß-1,3 glucanase act synergistically to strongly inhibit the growth of 15 out of 18 saprophytic and phytopathogenic fungi while the two enzymes individually do not affect the growth of all but two of the 15 fungi (Mauch et al., 1988b). Collectively, these results demonstrate that both enzymes are capable of inhibiting the growth of many different fungi and are thus anti-fungal in nature (Mauch et al., 1988b).

Chitinase and ß-1,3 glucanase induction is considered part of a general or non-specific defense response initiated in plants since induction can occur by non-fungal phytopathogens and various physical, chemical and environmental stresses. The evidence for this is vast, especially in reference to attack by fungal parasites.

Induction of ß-1,3 glucanase occurs in plants since ß-1,3 glucans, as well as ß-1,6 glucans, are endogenously present in plant cells. Callose, a ß-1,3 glucan, is present in sieve tubes, in cell wall appositions formed in response to wounding, and in primary plant cell walls (Boller, 1987). Constitutively produced cell wall ß-1,3 glucanase and those synthesized *de novo* and targeted to the wall in a defense response are thought to be important for turnover of callose and extension growth (Boller, 1987; Bowles, 1990).

Several studies of plant mediated ß-1,3 glucanase release of fungal elicitors involve investigations of soybean-*Phytophthora megasperma* f. sp. *glycenia* interaction. Two isoenzymes of ß-1,3 glucanase isolated from soybean cotyledons released high molecular weight glucomannan elicitors of glyceollin from cell walls and living hyphae of *P. megasperma* f. sp. *glycenia* (Keen et al., 1983). However, no direct fungitoxic activity on *P. megasperma* f. sp. *glycenia* by the purified soybean ß-1,3 glucanase could be detected (Yoshikawa et al., 1990). Exogenous treatment with ethylene caused an increase in ß-1,3 glucanase activity and partial resistance against the pathogen was conferred on susceptible soybean hypocotyls due to higher levels of glyceollin production.

This result supports the idea that constitutively produced ß-1,3 glucanase in soybean may cause resistance by elicitor-releasing activity rather than by direct inhibition of fungal growth. In soybean, ß-1,3 glucanase can be induced by treatment with mercuric chloride or infection with an avirulent fungus (Ham et al., 1991).

Chitinase induction seems to be unrelated with compatibility or incompatibility in host parasite interactions. Mauch et al. (1988a) demonstrated that growth of the nonpathogen of peas, *Fusarium solani* f. sp. *phaseoli* was as strongly inhibited by chitinase and ß-1,3 glucanase as the pathogen, *F. solani* f. sp. *pisi.* Both strains also induced chitinase and ß-1,3 glucanase to a similar extent in peapods. Chitinase induction occurs in susceptible cultivars of tomato challenged with *Verticillium albo-atrum* (Pegg and Young, 1981). Even fungi which do not contain chitin, such as the Oomycete *Peronospora tabacina* which causes blue mold in tobacco, can elicit chitinase in tobacco extracts (Pan et al, 1992). Systemically induced resistance in susceptible cultivars preinoculated with tobacco mosaic virus was correlated with the induction of two chitinase isozymes although induced resistance was elicited by two non-chitinous phytopathogens (Pan et al, 1992).

Chitinase induction can occur in plants in response to attack by viruses (Metraux and Boller, 1986; Bol et al., 1990; Garcia-Breijo et al., 1990; Nasser et al., 1990; Pan et al., 1992), bacteria (Metraux and Boller, 1986; Broekart and Peumans 1988) and many abiotic and physical stresses. This includes treatment with plant growth regulators such as ethylene (Boller et al., 1983; Boller and Vogeli, 1984; Mauch et al., 1984; Mauch and Staehelin, 1989; Kragh et al., 1991); auxin and cytokinin (Shinshi et al., 1987; Grosset et al., 1990; Kragh et al., 1991); kinetin (Esaka et al., 1990); mechanical injury or wounding (Mauch et al., 1988a); osmotic stress (Ohashi et al., 1985; Ohashi et al., 1987); necrotizing salt solutions (Metraux and Boller, 1986); chitosan (Mauch et al., 1984; Mauch et al., 1988a); phytoalexin elicitors (Mauch et al., 1984; Roby et al., 1986); pectic polysaccharides (Broekart and Peumans 1988); $HgCl_2$ (Nasser et al., 1990); 2,4-dichlorophenoxyacetic acid (2, 4-D) (Esaka et al., 1990; Kragh et al., 1991); CrO_3 (Jacobsen et al., 1992) and salicylic acid (Ohashi and Matsuoka, 1987; Ward et al., 1991).

Some purified plant chitinases can degrade substrates other than chitin. Several possess lysozyme (EC 3.2.1.17) activity in bean leaves (Boller et al., 1983); grains of wheat, barley and maize (Roberts and Selitrennikoff, 1988);

potatoes (Kombrink et al., 1988) and pea pods (Mauch et al., 1988a). Conversely, purified plant lysozymes such as those from fig, *Hevea brasiliensis*, latex of papaya, *Parthenocissus quinquifolia*, cultured *Rubus hispidus*, turnip roots and wheat germ have been shown to exhibit very high chitinase activity (Boller, 1987; Audy et al., 1990).

Studies of the active site of lysozyme reveal two carboxylic acid residues that participate in hydrolysis (Verburg et al., 1992). Based on the shared catalytic activity exhibited by lysozyme and chitinase, Verburg et al. (1992) reported that a basic chitinase (Chitinase A) from maize seed contains an essential tyrosine residue in the catalytic site. Although tyrosine is implicated in the glycosidic hydrolysis of chitinase as compared to acid catalysis by essential carboxylic acid residues of lysozyme, the authors do not rule out that chitinase and lysozyme share similar catalytic mechanisms. Two basic Class I chitinases from tobacco, CHN-A and CHN-B, contain certain proline rich residues which may be hydroxylated as a posttranslational mechanism as in other hydroxy-proline rich proteins to modulate chitinase activity and alter substrate specificity (Sticher et al., 1992).

Several transgenic plants have been created in order to study the inducible pattern of chitinase gene expression during fungal pathogen attack. Chitinase promoter sequences are fused with a ß-glucuronidase (GUS) reporter gene, tranformed into the desired plant and then assayed for GUS activity.

In transgenic *A. thaliana* containing a previously cloned and sequenced acidic Class III chitinase gene, GUS expression was induced in mesophyll cell surrounding lesions caused by *Rhizoctonia solani* infection and in transgenic tomato infected with *Alternaria solani* (Samac and Shah, 1991). However, infection with *Phytopthora infestans*, which contains only minor amounts of chitin in its hyphal cell walls, revealed the same pattern of gene expression. Therefore, activation of the promoter for the acidic chitinase in transgenic tomato plants by both pathogens was not limited to infection by chitin-containing fungi (Samac and Shah, 1991). Class III acidic chitinase accumulate locally and systemically in cucumber leaves inoculated with not only fungal but also viral pathogens causing a necrotic response (Metraux et al., 1988). Patterns of chitinase localization representing chitinase gene expression in infected tissues of different plants may be similar but cannot be strictly correlated with fungal parasite infection.

Constitutive expression of a bean endochitinase gene in transgenic

tobacco and canola plants afforded increased protection against disease caused by *R. solani* (Broglie et al., 1991). Fifty three percent of tobacco seedlings transplanted in *R. solani* infested soil without the 35-s chimeric gene died compared to a mortality rate of 22.7-37.1% for those with the gene (Broglie et al., 1991) and similar results were found in canola infected with *R. solani.* However, increasing chitinase activity in transgenic plants by overexpression in tobacco has no discernible effect on resistance by the fungal pathogen *Cercospora nicotinae* (Neuhaus et al., 1991a). Broglie et al. (1991) speculate that modification of chitinase expression alone is not sufficient to provide protection against a wide range of chitinous phytopathogens.

Modified microorganisms can be used as effective biological control agents to confer potential plant antifungal resistance in addition to the prospect of creating transgenic plants. A 62% reduction of bean disease caused by *S. rolfsii* and cotton disease caused by *R. solani* occurred by spraying crops with a partially purified endochitinase cloned from *Serratia marcescens* into *E. coli* and with viable cells of transformed *E. coli* themselves (Shapira et al., 1989). Hyphal tip bursting was observed by light and scanning electron microscopy (Shapira et al., 1989). The *S. marcescens* endochitinase (ChiA) gene was cloned into the plasmid vector pBR322 with a bacteriophage λ operator and used to produce large quantities of the enzyme in *E. coli*.

The Lectin-Chitinase Debate

Mirelman et al. (1975) were the first to speculate that plant lectins may act as natural anti-fungal agents because of the inhibition of growth and spore germination of *Trichoderma viride* with WGA specific for oligomers of GlcNAc. However, re-evaluation of these results proved that WGA, when prepared by affinity chromatography with a chitobiose column; also specific for chitinase, was contaminated with chitinase and responsible for the inhibitory effect on *T. viride* and not the lectin itself (Schulbaum et al., 1986). The anti-fungal role of plant lectins remained unresolved until a chitin-binding lectin was isolated from rhizomes of the stinging nettle, *Urtica dioica* or *U. dioica* agglutinin (UDA). This lectin was not only chitinase free but also a potent inhibitor of the growth of several phytopathogenic and saprophytic chitin containing fungi *in vitro* (Brokaert et al., 1989). Moreover, UDA was free of chitosanase, ß-1,4 N-acetylglucoaminidase and ß-1,3 glucanase activity. UDA shows a striking

amino acid sequence homology with WGA, but has a molecular mass of 8.5 kDa as compared to 36 kDa and is unusually heat and acid resistant. Compared to inhibitory bio-assays with several plant chitinases, UDA did not cause lysis at the hyphal tips or affect spore germination which suggests that the inhibitory mechanism of UDA differs from chitinase (Brokaert et al., 1989).

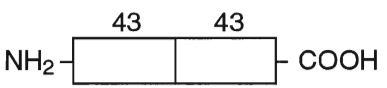
Many plant proteins share a common 43 amino acid chitin-binding domain (Chrispeels and Raikhel, 1991); WGA, rice and barley lectins have four of these chitin-binding domains. A protein termed hevein isolated from the phloem sap of the rubber tree exhibits chitinase and lysozyme activity, is anti-fungal *in vitro* and has one chitin-binding domain (Rozeboom et al., 1990; Van Parijs et al., 1991). The large subunit of a killer toxin encoded by a plasmid pGKL1 of *Kluveromyces lactis* which causes irreversible growth inhibition in sensitive yeast cells has one putative chitin binding domain and similarity to an exochitinase from *Serratia marcescens* (Bradshaw, 1990). This large subunit may arrest the growth of the target yeast cells by interfering with cell wall biosynthesis and has chitinase activity (Bradshaw, 1990; Butler et al., 1991). Wound induced genes in potato tubers, *win* 1 and *win* 2 encode for proteins with a highly conserved 43 amino acid domain (Bowles, 1990). A chitinase from *R. oligosporus* and the chitinase from *S. cerevisiae* responsible for cell separation both have one putative chitin-binding domain (Kuranda and Robbins, 1991; Yanai et al., 1992) and several Class I plant chitinases also contain such a domain (Shinshi et al., 1990).

The stinging nettle lectin cDNA has been cloned and sequenced and shown to contain an open reading frame encoding 374 amino acids even though UDA is a 8.5 kDa protein and consists of two 43 amino acid chitin-binding domains (Lerner and Raikhel, 1992). Amino acid sequence studies revealed a putative signal sequence in the amino terminal, two chitin binding domains, a small spacer region and a long carboxyl terminal domain (Fig. 4). Intriguingly, 60% sequence homology was found with the carboxy terminal domain and the catalytic domains of several cloned chitinases. Thus, the nettle lectin gene encodes both a lectin and a chitinase. Presumably, UDA undergoes a post translational processing event to release the chitin binding domains from the catalytic chitinase domain hence the 8.5 kDa molecular mass found during isolation. The spacer region in the stinging nettle lectin preprotein may contain the recognition site for proteolytic cleavage (Lerner and Raikhel, 1992). Class I chitinase preproteins do not undergo post translational cleavage of the carboxy

Fig. 4. Model of stinging nettle lectin, *Urtica diocia* agglutinin (UDA) primary preprotein structure derived from cDNA clones. The preprotein (374 amino acids) undergoes a post-translational processing event to release the chitin-binding domain from the chitinase 'like' catalytic domain yielding a mature 8.5 kDa protein (86 amino acids) with only two chitin binding domain structure. Redrawn with permission from Lerner and Raikhel (1992).

UDA preprotein

UDA mature protein



4

= Signal sequence

= Chitin binding domain

= Spacer region

= Chitinase 'like' catalytic domain

terminal whereas UDA, other lectins and hevein do. This result is significant in light of the antifungal nature of UDA; the lectin and chitinases differ in their mode of *in vitro* inhibition of fungal growth (Brokaert et al., 1989). Since it is not clear whether overexpression of chitinase in transgenic plants has an effect on fungal resistance, expression of UDA in transgenic plants may enhance resistance to fungal pathogens where chitinase does not and may provide an opportunity to examine any synergistic anti-fungal effect of the lectin and the chitinase (Lerner and Raikhel, 1992).

Materials and Methods

Organisms and Cultural Conditions

Cultures of the mucoraceous fungi, *Choanephora cucurbitarum* (Berk & Rav.) Thaxter, *Phascolomyces articulosus* Boedijn ex. Benny and Benjamin, *Mortierella pusilla* Oudeman, and *Mortierella candelabrum* v. Teigh and Le Monn were routinely maintained on a medium containing malt extract 20 g, yeast extract 2 g, agar 20 g (MYE) supplemented with salts of the microelements Fe^{2+} ($FeCl_2$) 0.2 g, Mn^{2+} ($MnCl_2$) 0.2 g, Zn^{2+} (ZnSO4) 0.4 g and vitamins thiamine 0.2 g and biotin 0.05 g in 1 L distilled water adjusted to pH 6.5 (Phipps and Barnett, 1975). These fungi are used in our laboratory for studies on host-parasite interactions as susceptible and resistant hosts or nonhost to the biotrophic haustorial mycoparasite, *Piptocephalis virginiana* Leadbeater and Mercer which is also a member of Zygomycetes (see Manocha, 1987). The latter was maintained on its susceptible host *C. cucurbitarum* in a two member culture grown in complete darkness which is known to inhibit sporulation of the host and not the mycoparasite (Manocha, 1985). Asexual sporangiospores of each fungus were liberated by adding sterile distilled water to the culture plate followed by gentle scraping of the surface with a glass rod.

Spores of each fungus (10^6 spores/ml) were grown in MYE liquid medium (same composition as above but minus agar), at 22±1°C with agitation on a gyrotary shaker (New Brunswick Scientific, New Brunswick, NJ) at 100 rpm. Each of these fungi germinated at different times: *C. cucurbitarum* 3-4 h, *P. articulosus* 8-10 h, *M. pusilla* 10-12 h, *M. candelabrum* 4-5 h and *P. virginiana* 18-20 h. Following germination, the spores were washed twice by gentle centrifugation with 0.01M phosphate buffered saline (PBS) pH 7.2 and resuspended in PBS for further use. Growth was also carried on a semi-solid medium in 5 cm agar plates overlayed with sterile dialysis membranes (Spectrum Medical Industries Inc. Los Angeles, CA) inoculated in the center with 10 µl of spore suspension containing 10^6 spores/ml. For host-parasite interaction studies, a ratio of at least 10:1 parasite:host spores (eg. $10^7/10^6$ spores/ml) was inoculated in the center of the agar plates. These cultures were incubated for 20-22 h at 22±1°C and infection structures were verified by light microscopy. Mycelia from liquid cultures and from the surface of the dialysis membranes were gently washed with PBS buffer.

Chemicals

The following comprises a list of the source of all the chemicals used in this study. All culture media: Difco Laboratories (Detroit, MI); Nitrocellulose membranes and molecular biology reagents and equipment: Bio-Rad Laboratories (Richmond, CA); alkaline phosphatase conjugated Fc fragment specific goat-anti rabbbit IgG (AP-GAR), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT): Promega Corporation (Madison, WI); S&S Affinica Protein A starter kit: Schleicher and Schnell Inc. (Keene, NH); Fluorescein isothiocyanate conjugated goat anti-rabbit Fc fragment specific IgG (FITC-GAR): Jackson Immunoresearch Laboratories (West Grove, PA); WGA, FITC-WGA, *Phytolacca americana* agglutinin (PAA) with specificity for N, N'-diacetyl-chitobiose, lysozyme, Bovine Serum Albumin Fr. V. (BSA), Proteinase K, Pronase E, Trypsin and all other chemicals: Sigma Chemical Co., (St. Louis, MO); Intercellular fluid extracts (ICF) of tobacco and barley were a kind gift of Dr. Alain Asselin, Universite Laval, St. Foy, Quebec, Canada. The protocol for isolation of the ICF's is described in Grenier and Asselin (1990).

Isolation, Purification and Assay of Microsomal Chitinase

Protocols outlining isolation and purification of microsomal and cytosolic fractions of chitinase of *C. cucurbitarum* and *P. articulosus* are described in Balasubramanian and Manocha (1992).

Protein Extraction

The method of Penefsky and Tzagoloff (1971) was followed. Mycelia of *C. cucurbitarum* grown for 24 h in MYE liquid medium were filtered through a Whatman filter paper No.1 in a Buchner funnel, washed and immediately plunged into liquid nitrogen. Frozen mycelium was broken in a mortar and pestle with a small amount of sterile sea sand and TEPI buffer consisting of 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF),1 mM iodoacetic acid, pH 6.8 at 4°C . The crude mixture was then centrifuged at 1,000 rpm (IEC 870) at 4°C for 10 min and the supernatant was collected. One part cold n-butanol was then added and sample was respun at 10,000 rpm (IEC 870) at 4°C for 10 min to separate

the protein and lipid phases. The protein phase was collected and dialysed in TE (TEPI without PMSF and iodoacetic acid) pH 7.5 and then lyophilized and resuspended in minimal amount of TEPI buffer.

Preparation of Antisera

140 µg of purified microsomal chitinase obtained from *C. cucurbitarum* was dissolved in 500 µl sterile distilled water from which 250 µl was mixed with an equal volume of Freund's complete adjuvant and the mixture was injected into a New Zealand White rabbit at multiple subcutaneous sites. The remaining sample was mixed with an equal volume of Freund's incomplete adjuvant and the mixture was re-injected subcutaneously after 2 weeks. Bleedings were made 2, 4, 6 and 8 weeks after the last injection. Following clotting, immunoglobulins were separated according to the method described by Hurn and Chantler (1980). Protein concentration was calculated to be 1.34 mg/ml using the Bradford method (1976) and Bio-Rad dye concentrates. The antiserum was purified further by an affinity chromatography kit consisting of a 1 ml cartridge containing Protein A covalently linked to a stable, synthetic gel matrix. Following regeneration of the Protein A cartridge with a neutral pH buffer, the sample was bound to the Protein A ligand with a high pH binding buffer and eluted with a low pH elution buffer and immediately resuspended in 0.01 M PBS pH 7.2.

Dot-immunobinding Procedure

Dot-immunobinding assays were performed according to the method of Parent et al., (1985). Strips of nitrocellulose membrane (0.45 µm pore size) were wetted by capillarity in 20 mM Tris, 500 mM NaCl (TBS) buffer pH 7.5 and dried. Two µl of protein samples (1 mg/ml) were applied as dots and allowed to dry at room temperature. The strips were then: (i) blocked by immersion in 3% (W/V) gelatin in TBS for 1 h; (ii) washed once in TTBS i.e.TBS plus 0.05% Tween 20 (V/V) for 5 min; (iii) immersed in antiserum diluted 1:10 000 in TTBS for 1 h; (iv) three rinses of TTBS for 30 min each; (v) treated with AP-GAR diluted 1:10,000 in TTBS for 1 h; (vi) two washes of TTBS for 5 min each and once with TBS for 5 min; (vii) stained for protein detection with 0.03% BCIP (W/V) and 0.06% NBT (W/V) in 0.1 M TBS pH 9.5 (containing 1 mM $MgSO_4$) and

incubated under total darkness until color development. Controls consisted of (i) omission of the primary antiserum (ii) omission of the secondary antibody and (iii) replacement of the primary antiserum with pre-immune serum.

Western Blot

Solutions of 1 mg/ml of each of crude *C. cucurbitarum* protein extract; WGA; PAA; tobacco ICF and barley ICF were dissolved in a standard 2% Sodium Dodecyl Sulfate (SDS) reducing buffer for 4 min at 95ºC. Protein sample (15 µg of each) was loaded onto a Bio-Rad mini SDS-PAGE gel apparatus consisting of an 11% acrylamide separating gel and a 4% stacking gel and electrophoresed at 150 V (constant voltage) for approximately 45 min.

Western blots were performed using the method described by Towbin et al., (1979). Proteins separated by SDS-PAGE were transfered to nitrocellulose membranes using the Bio-Rad Trans Blot Apparatus and transferred overnight in a transfer buffer consisting of 25 mM Tris, 192 mM glycine and 20% (V/V) methanol, pH 8.3 at 30 V (constant voltage). The procedure for detection of proteins and controls experiments were the same as used for dot immuno-binding assay.

Immuno-fluorescence

An indirect immuno-fluorescence protocol using FITC-GAR capable of binding to rabbit-anti-chitinase was chosen. 200 µl aliquots of the germinated spores of each fungus was placed in sterile eppendorf tubes and briefly washed with PBS to form a small pellet. These cells and mycelia from liquid medium cultures or those formed on the surface of the solid medium were all immediately fixed with 2% formaldehyde in 0.01M PBS for 2 h at room temperature followed by thorough washing with PBS. Appropriate dilutions, incubation time and temperature of the primary and secondary antisera which gave no further change in fluorescence intensity were empirically determined for ideal results. Cells were incubated with 1:10 anti-chitinase for 1 h at 4ºC followed by careful washing three times for 5 min each with PBS plus 4% bovine serum albumin (BSA) plus 1% thimerosal with brief centrifugation to remove all unbound primary antiserum. A 1:10 dilution of the FITC-GAR was added for 30 min at room temperature and washed with PBS-BSA followed by

PBS buffer alone. The cells were carefully mounted on clean glass slides and viewed with a Wild-Leitz DIAPLAN epifluorescence microscope equipped with an automatic camera. Controls consisted of (i) incubation with rabbit anti-chitinase only (ii) incubation with FITC-GAR only (iii) replacement of the rabbit anti-chitinase with rabbit pre-immune serum.

Treatment of the germinated cells with commercially available proteinases prior to incubation with anti-chitinase is described in Manocha and Sahai (1991). A suspension of 1 ml of germinated cells and 1 ml of the proteolytic enzymes, Pronase E (11.6 units/ml), Proteinase K (13.8 units/ml) or Trypsin (14.4 units/ml) prepared in PBS and adjusted to the respective pH optimum (pH 6.8 for Pronase E and pH 8 for Proteinase K and Trypsin) were incubated for 30 min at room temperature. Permeabilization of the cell surface prior to treatment with anti-chitinase was performed by immersion of the cells in methanol kept at -20°C for 4 min followed by acetone kept at -20°C for 1 min. Mechanical injury consisted of carefully cutting hyphae grown on the agar surface with a razor blade prior to fixation. The entire indirect immuno-fluorescence assay was repeated at least three times.

Direct FITC-WGA Binding Assay

The protocol outlined in Manocha (1985) was used. Washed cells were incubated with 7 ml of FITC labelled wheat germ agglutinin (1 mg/ml) and 43 ml of 0.01M PBS plus 6% BSA and 1% thiomersal for 30 min at room temperature in complete darkness. The cells were then extensively washed in PBS plus 1% thiomersal and mounted on clean glass slides and viewed with fluorescence microscopy. The control consisted of preincubation of WGA with 100 mM N, N', N" tri-acetylchitotriose prior to addition of the cells. This direct FITC-WGA binding assay was also repeated at least three times.

Results

Dot Immunoblot

Specificity of the anti-chitinase antiserum was initially tested using a dot immunoblot protocol as described under Material and Methods (Fig. 5i & ii). Cross reactivity was found with purified microsomal chitinase isolated from *C. cucurbitarum* (Fig. 5A). A reaction was also found with the cytosolic chitinase from *C. cucurbitarum* (Fig. 5B) and microsomal and cytosolic chitinase from *P. articulosus* (Fig. 5C and D, respectively). Strong cross reactivity was found with commercially available chitinase from *Streptomyces* sp. (Fig. 5E) while a weak reaction was observed with a crude 10 day culture filtrate extract from a chitinase secreting *Streptomyces* sp. strain (Fig. 5F). Fairly strong cross reactivity was found with both chitin binding lectins WGA and PAA to a lesser extent (Fig. 5H and I); both plant ICF's from tobacco and barley (Fig. 5J and K) and also lysozyme (Fig. 5L). There was no reaction with BSA (Fig. 5G) or with the control experiment omitting the anti-chitinase antiserum (5ii) or the secondary antibody.

Western Blot

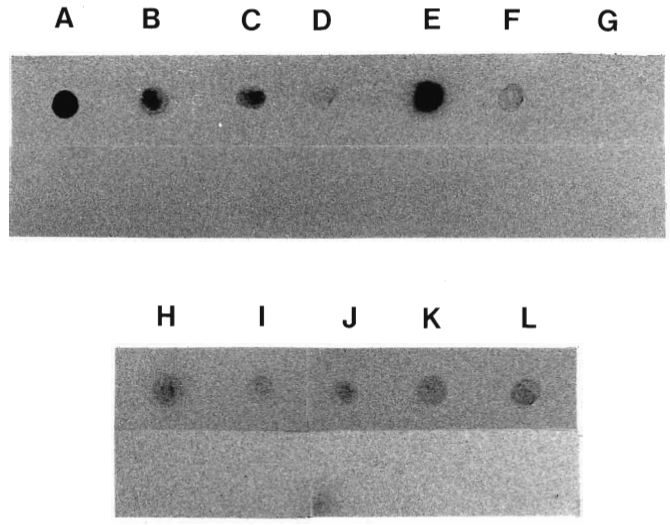
Two prominent bands (Mr 98 and 66 kDa) and one faint band (97 kDa) were observed (Fig. 6A) with the crude protein extract of *C. cucurbitarum.* The band with molecular mass 66 kDa corresponds to microsomal chitinase isolated from *C. cucurbitarum* (Balasubramanian and Manocha, 1992). Cross reactivity of the anti-chitinase antiserum was also seen with PAA and WGA with discrete bands corresponding to molecular masses of 44 kDa and 22 kDa, respectively (Fig. 6B and C). ICF's of Tobacco showed several bands most notably at 27 kDa (Fig. 6D) and 26 kDa with barley (Fig. 6E). Specificity of the anti-chitinase antiserum was verified with three controls described under Materials and Methods, none of which gave any reaction (data not shown).

Fig. 5i & ii. Dot immunoblot with anti-chitinase antiserum (5i). Chitinase isolated from microsomal (A) and cytosolic (B) fractions of *Choanephora cucurbitarum.* Microsomal (C) and cytosolic (D) chitinase isolated from *Phascolomyces articulosus.* Commercial chitinase from *Streptomyces* sp. strain (E) (SIGMA). Crude 10-day culture filtrate extract from chitinase secreting *Streptomyces* sp. strain (F). BSA (G), WGA (H), PAA (I), ICF's from tobacco (J) and barley (K) and lysozyme (L). The control experiment deleting the anti-chitinase antiserum and incubating with the secondary antiserum alone is shown in 5ii. See text for abbreviations.

Fig. 6. Western blot with anti-chitinase antiserum. Crude protein extract from *C. cucurbitarum* (A), PAA (B), WGA (C) and ICF's from tobacco (D) and barley (E).The masses (kDa) of the molecular weight standards are shown in the leftmost lane.

5 i

ii



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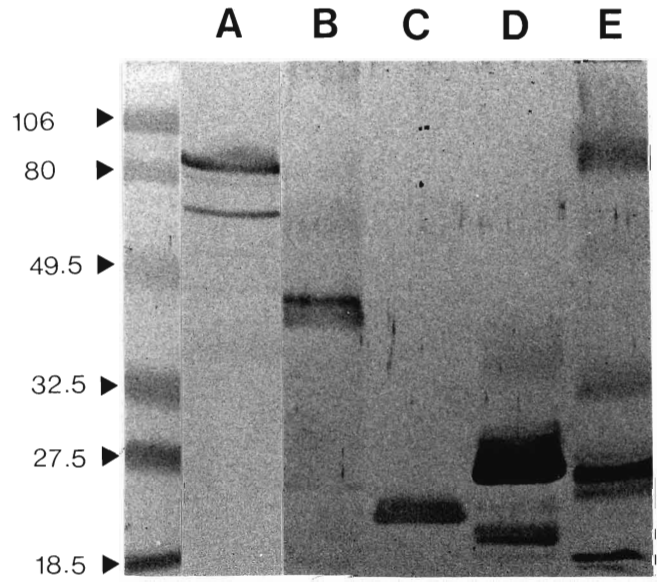


Part A. Chitinase Immuno-fluorescence of Five Zygomycetous Fungi

Results of the indirect anti-chitinase fluorescence immuno-cytochemistry and direct FITC-WGA binding assay are summarized in Table 2. There was no fluorescence in the ungerminated spores of *C. cucurbitarum, M. pusilla* and *M. candelabrum* with the anti-chitinase probe. However, a positive reaction for chitinase was seen in the ungerminated spores of *P. articulosus* and *P. virginiana* with strong fluorescence along the perimeter of the spore in a slightly discontinuous fashion for *P. articulosus* (Fig. 7A & B) and weak fluorescence along the spore perimeter of *P. virginiana* (Fig. 8A & B).

Chitinase was also present along the perimeter of the swollen spores of all the fungi tested. Strong fluorescence in *P. virginiana* is represented in Figure 9 (A & B) while weak fluorescence in this manner is shown in *M. pusilla* (Fig. 10A & B). There was no spore swelling in the case of *C. cucurbitarum.*

Germination of *C. cucurbitarum* occurred by a lengthwise splitting of a sporangiospore striation during the outward pushing of the newly emerging germ tube. Chitinase was found to be localized only at the point of germ tube emergence with slight fluorescence along the perimeter of the spore. There was no localization of chitinase at the apex nor was there any detection of fluorescence at newly forming branch sites. All five fungi had the same pattern of fluorescence during germination when probed by the anti-chitinase antibody. Such a result is clearly illustrated with *C. cucurbitarum* (Fig. 11A & B). Mild proteolysis with commercially available proteinases and permeabilization of the cell surface failed to reveal chitinase at the apex or at the newly emerging branch site.

There was a lack of fluorescence with anti-chitinase in 24-h-old hyphae of *C. cucurbitarum, M. pusilla, M. candelabrum* but not *P. articulosus* and *P. virginiana.* Reiteration of the lack of fluorescence at the hyphal apex was seen when mechanically injured hyphae of *M. pusilla* showed fluorescence while apices of the same hyphae did not show any fluorescence (Fig. 12A & B).

Sporangia developed in each fungus within 48 h on the agar surface. and strong fluorescence was seen along the perimeter of the sporangium as illustrated in *M. pusilla* (Fig. 13A & B).

There was no fluorescence with deletion of either the anti-chitinase antiserum or FITC-GAR and with replacement of anti-chitinase with pre-immune serum. Cells in each of the cases exhibited only slight autofluorescence.

Table 2

Summary of chitinase immuno-fluorescence (A) and FITC-WGA (B) binding patterns at various growth stages of zygomycetous fungi

Growth stage	*C. cucurbitarum*		*M. pusilla*		*M. candelabrum*		*P. articulosus*		*P. virginiana*	
	A	B	A	B	A	B	A	B	A	B
Ungerminated spore	-	-*	-	-	-	-	++[a]	++[a]	+[a]	++[a]
Swollen spore	ND	ND	+[a]	++[a]	+[a]	+[a]	++[a]	++[a]	++[a]	++[a]
Germ tube	++[b]	++[c]	++[b]	++[c]	++[b]	++[b]	++[b]	++[c]	++[b]	++[a]
Hyphae	-	+[d]	-	++[a]	-	+[d]	++[a]	++[a]	++[a]	++[a]
Sporangium	++[a]	++[a]	++[a]	++[a]	++[a]	++[a]	++[a]	++[a]	ND	ND

* Intensity of Fluorescence: - no fluorescence, + weak fluorescence, ++ strong fluorescence, ND not determined.

[a] fluorescence along entire perimeter of cell surface

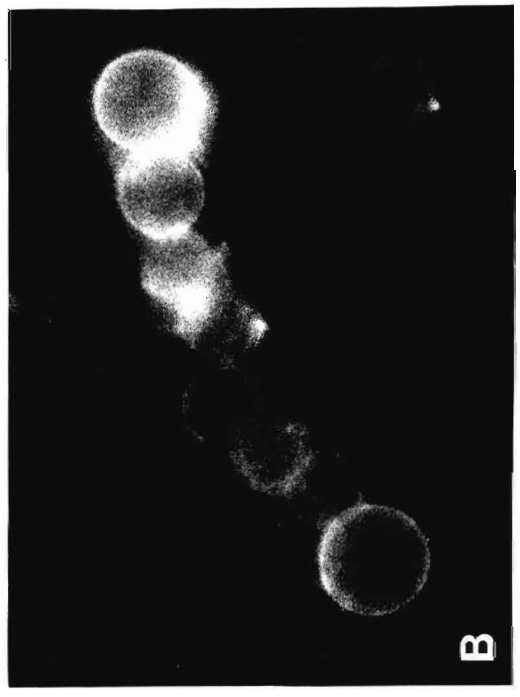
[b] fluorescence along spore perimeter and site of germ tube emergence

[c] fluorescence along spore perimeter, site of germ tube emergence and at the apex

[d] fluorescence of some hyphae at the apex

Figs. 7-13. Representative bright field, phase contrast and fluorescence micrographs of indirect **anti-chitinase immunofluorescence** test of five zygomycetous fungi. See text for explanations.

Figs. 7 & 8. Ungerminated spores of *P. articulosus* (Fig. 7A & B) and *P. virginiana* with indirect anti-chitinase immunofluorescence test (Fig. 8A & B) X 1200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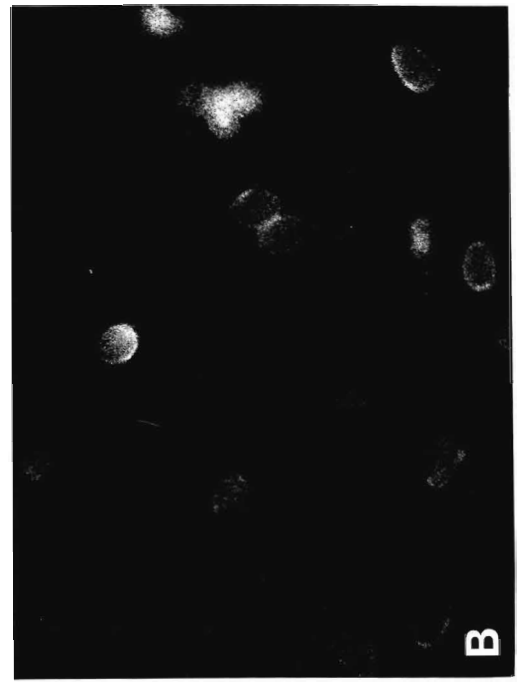
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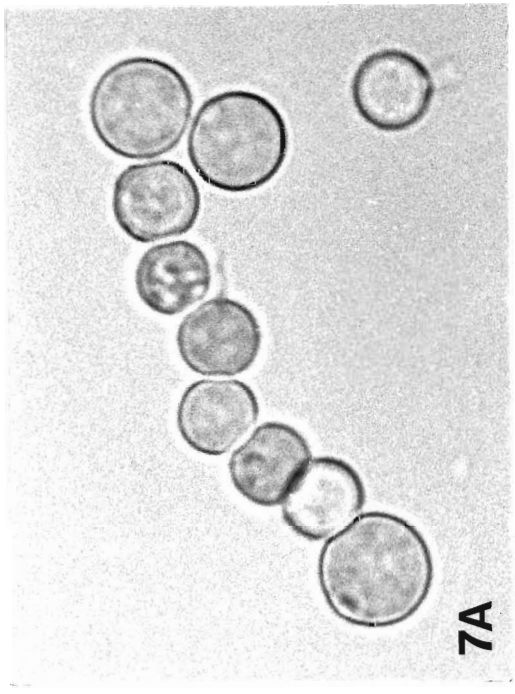
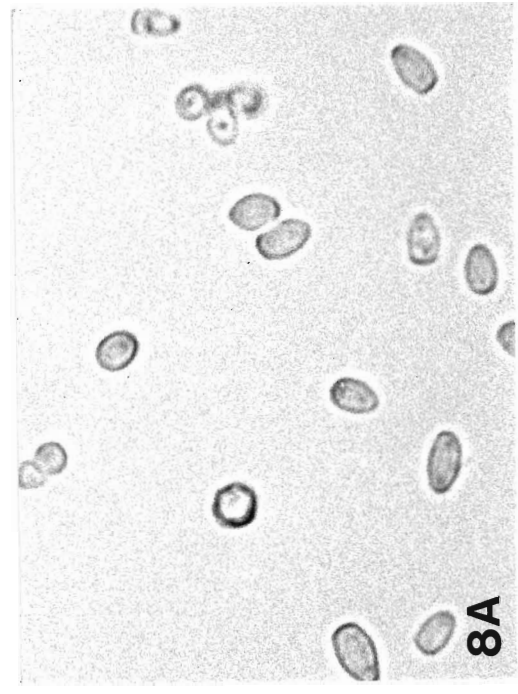
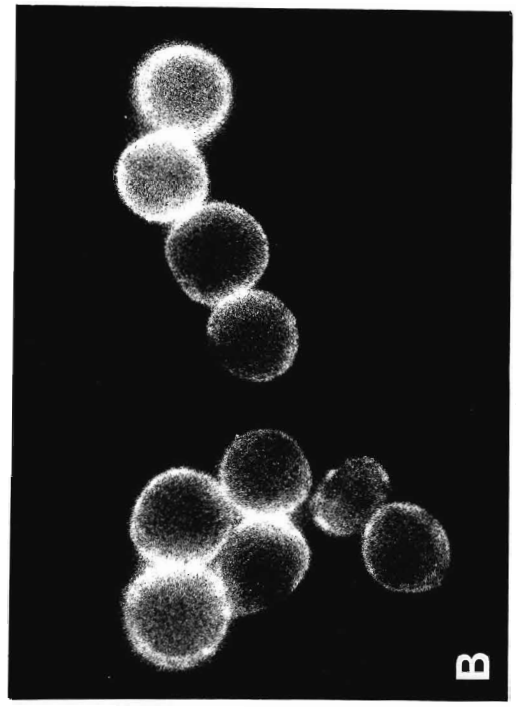

7A


8A

Figs. 9 & 10. Swollen spores of *P. virginiana* (Fig. 9A & B) and *M. pusilla* with indirect anti-chitinase immunofluorescence test (Fig. 10A & B) X 1200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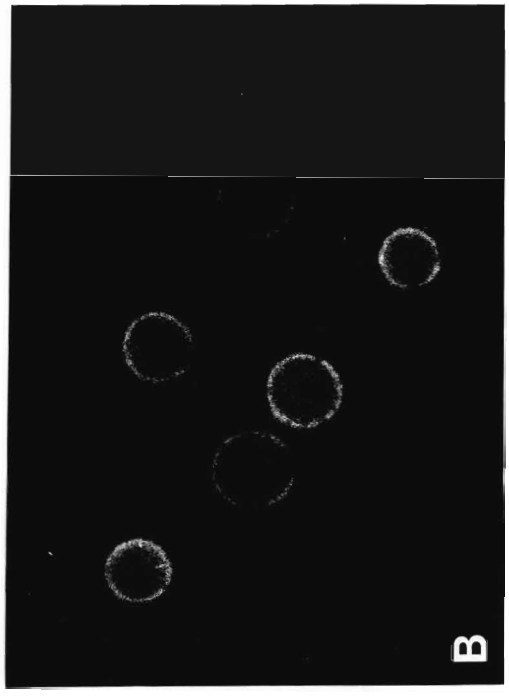
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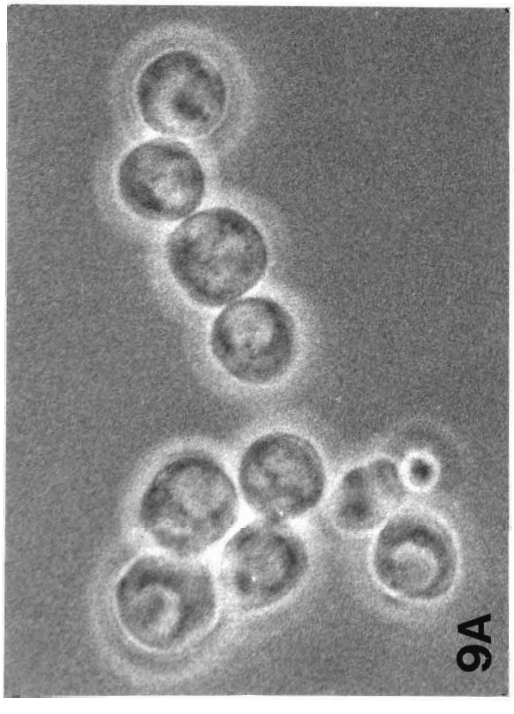

9A

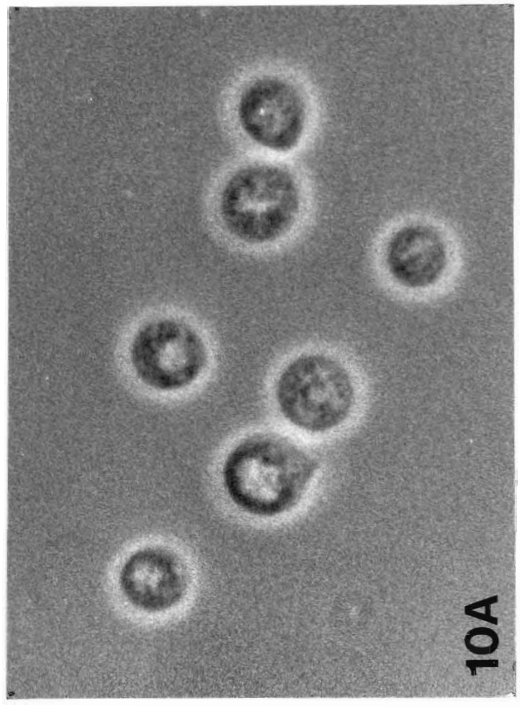
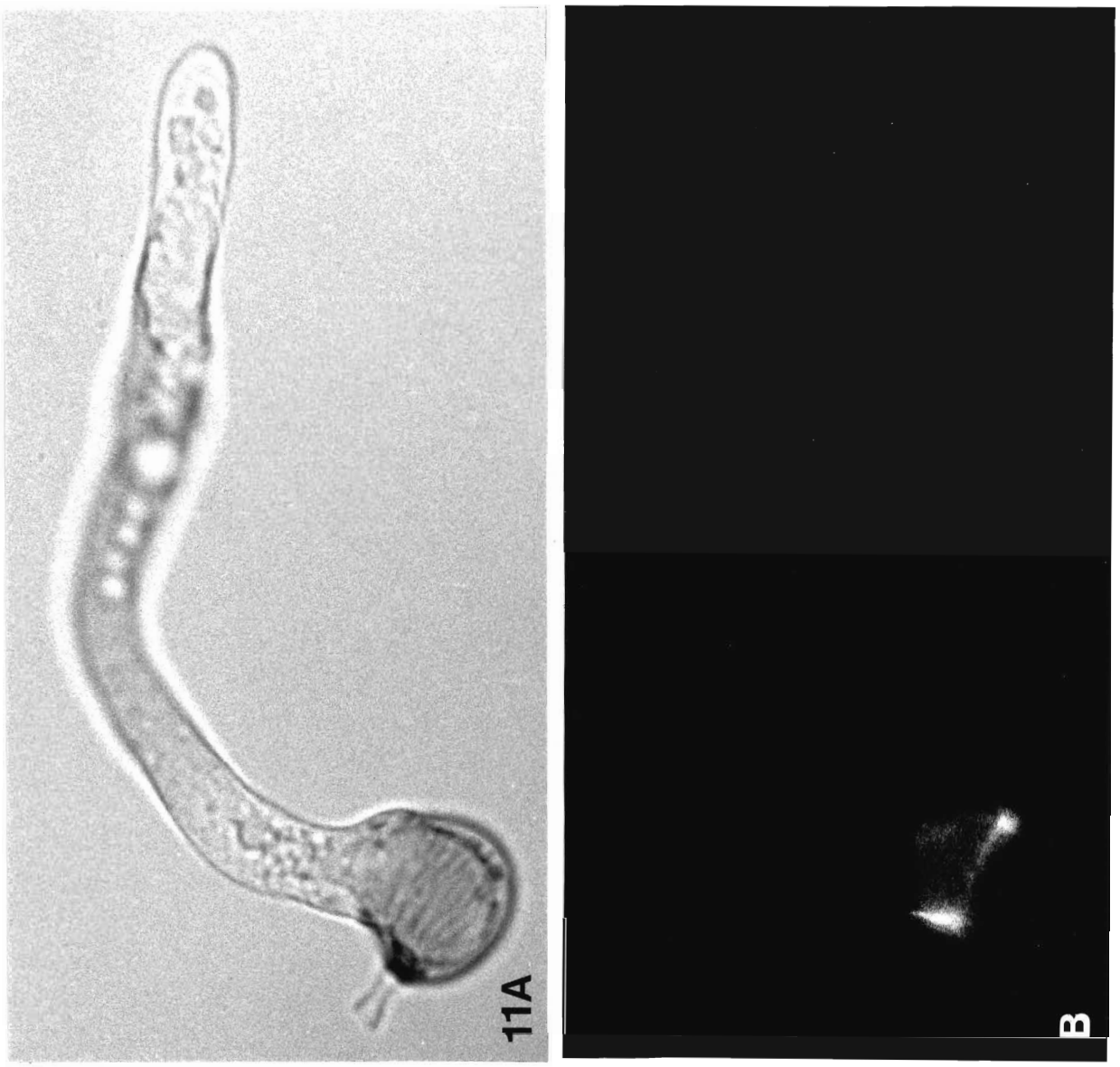

10A

Fig. 11A & B. A germinated spore of *C. cucurbitarum* with indirect anti-chitinase immunofluorescence test X 1200


11A
B

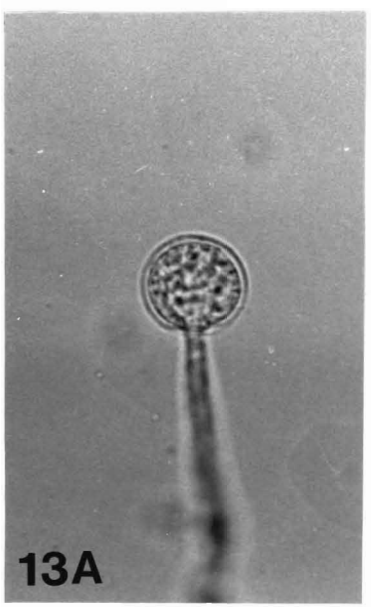
Figs. 12 & 13. Mechanically injured hyphae (Fig. 12A & B) X 1500 and a sporangium (Fig. 13A & B) X 600 of *M. pusilla* with indirect anti-chitinase immunofluorescence test

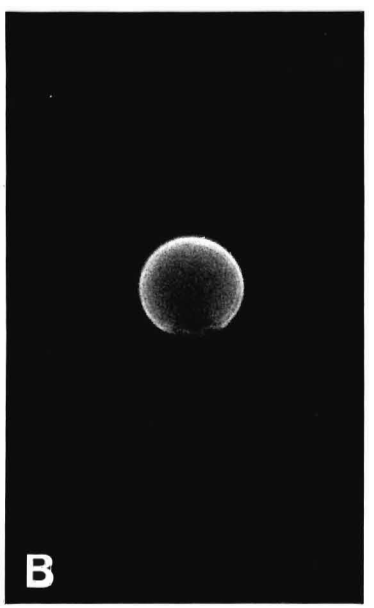
Figs. 14-20. Representative bright field and fluorescence micrographs of the **direct FITC-WGA binding assay** of five zygomycetous fungi. See text for explanations.

Fig. 14A & B. A germinated spore of *C. cucurbitarum* treated with FITC-WGA X 600

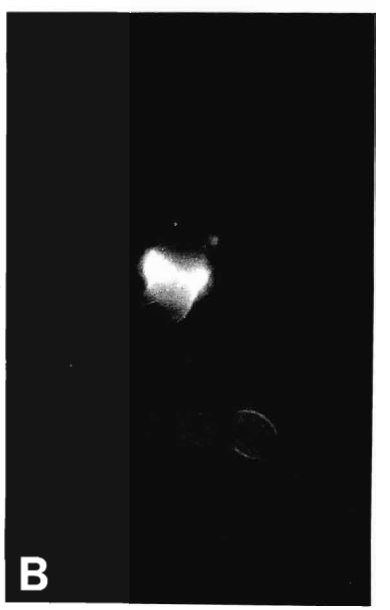

12A


B


13A


B


14A


B

Part A. Direct FITC-WGA Binding Assay of Five Zygomycetous Fungi

Just as the case with the anti-chitinase probe, oligomers of GlcNAc were not localized in ungerminated spores of *C. cucurbitarum, M. pusilla* and *M. candelabrum,* but strong fluorescence was observed along the perimeter of *P. articulosus* and *P. virginiana.* Swollen spores of each fungus also matched the result with anti-chitinase in that fluorescence was seen along the spore perimeter.

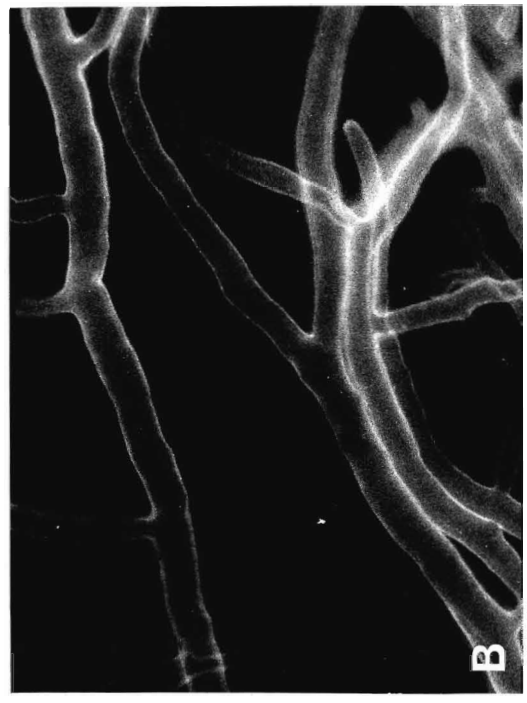
Three reactions in the germinated spores were found with FITC-WGA treatment. Most notably was the localization of oligomers of GlcNAc at the hyphal apex in addition to fluorescence at the site of germ tube emergence and the perimeter of the spore as shown in *C. cucurbitarum* (Fig. 14A & B). The same pattern was found with *M. pusilla* and *P. articulosus* while in *M. candelabrum* there was no difference between chitinase localization and binding with FITC-WGA (Fig. 15A & B). In *P. virginiana,* fluorescence was seen along the entire perimeter of the germinated spore.

Twenty four-h-old hyphae of *M. pusilla* and *P. articulosus* exhibited strong fluorescence along the entire hyphal cell surface (Fig. 16A & B), while hyphae of *C. cucurbitarum* showed fluorescence at the hyphal apex and newly emerging branch sites when treated with FITC-WGA (Fig. 17A & B). With *M. candelabrum,* there was apical and subapical fluorescence (Fig. 18A & B).

Mechanically injured hyphae matched the result found with the anti-chitinase probe with fluorescence present at the site of injury (Fig. 19A & B). Sporangia of each fungus also showed strong fluorescence along the perimeter as was the case with chitinase localization (Fig. 20A & B). Pretreatment of FITC-WGA with its inhibitory hapten prior to addition of the cells resulted in slight autofluorescence.

Figs. 15 & 16. A germinated spore of *M. candelabrum* (Fig. 15A & B) X 1200 and twenty four-h-old hyphae of *P. articulosus* (Fig. 16A & B) treated with FITC-WGA X 600


B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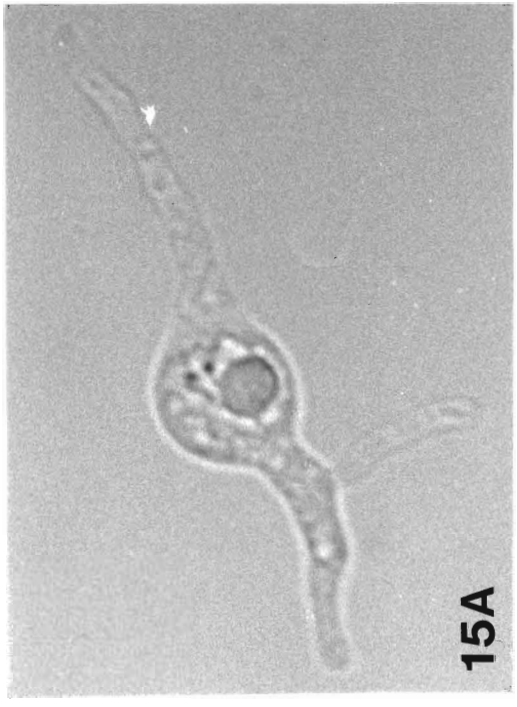
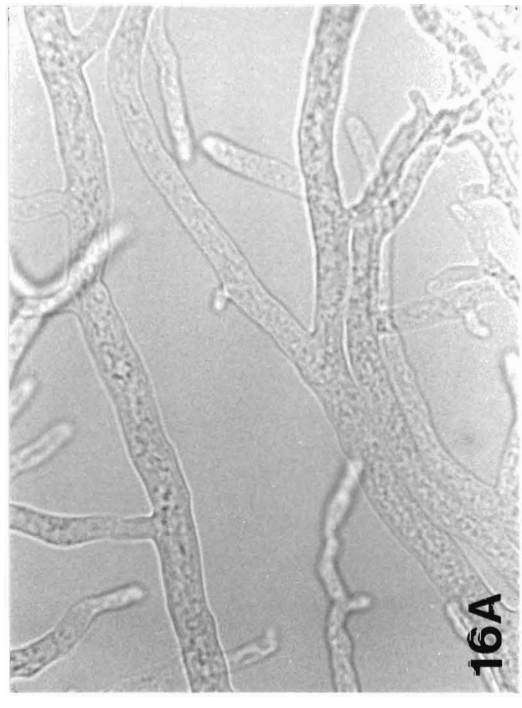
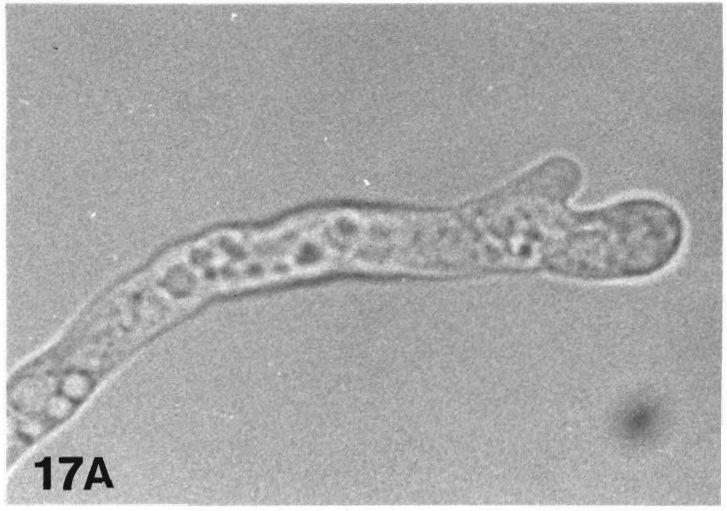

15A


16A

Figs. 17 & 18. Twenty four-h-old hyphae of *C. cucurbitarum* (Fig. 17A & B) X 1200 and *M. candelabrum* (Fig. 18A & B) treated with FITC-WGA X 600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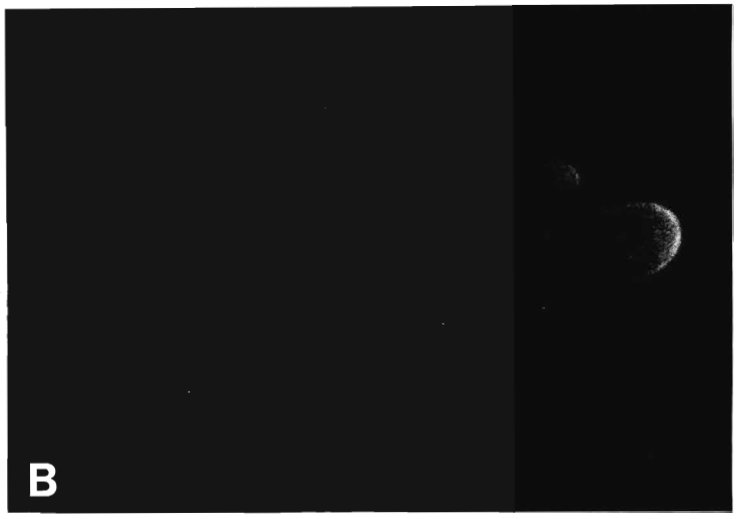
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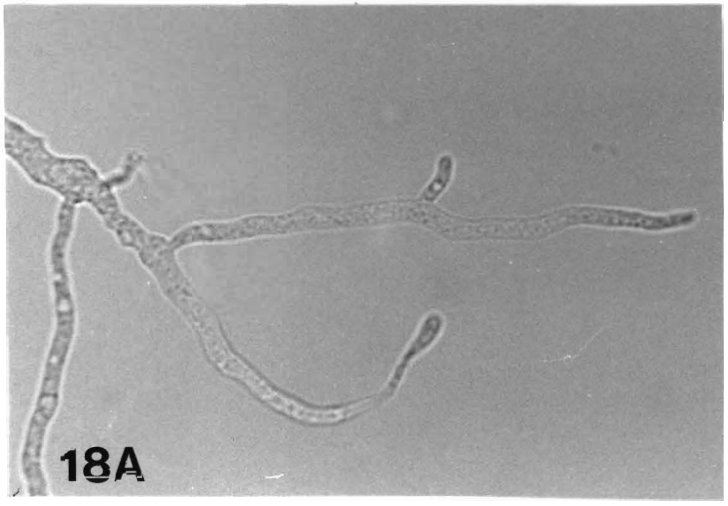
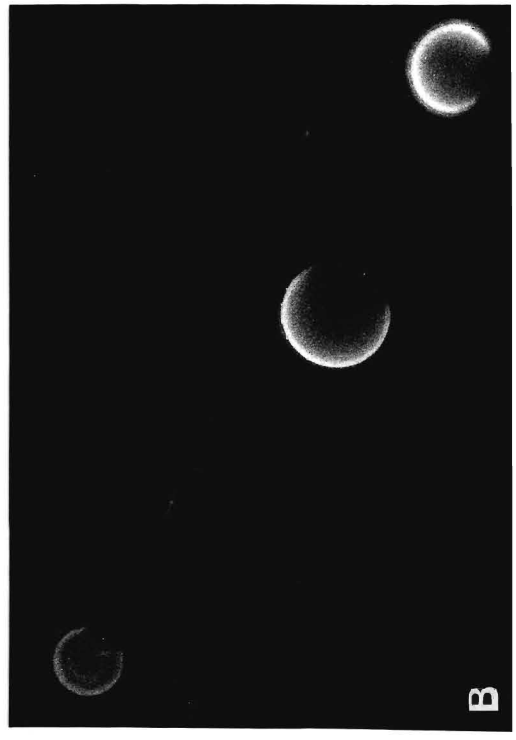

18A


B

Figs. 19 & 20. Mechanically injured hyphae of *C. cucurbitarum* (Fig. 19A & B) X 1200 and several sporangia of *M. pusilla* (Fig. 20A & B) treated with FITC-WGA X 600


B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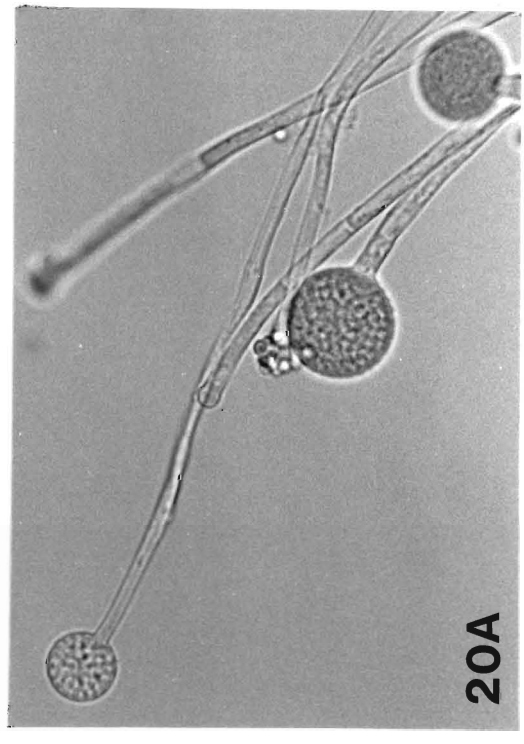
19A

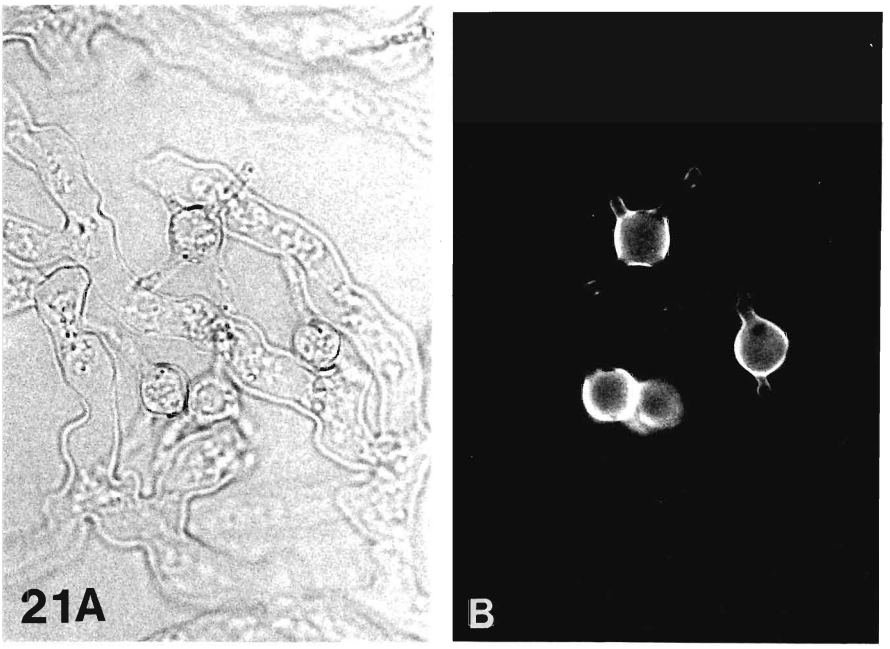

20A

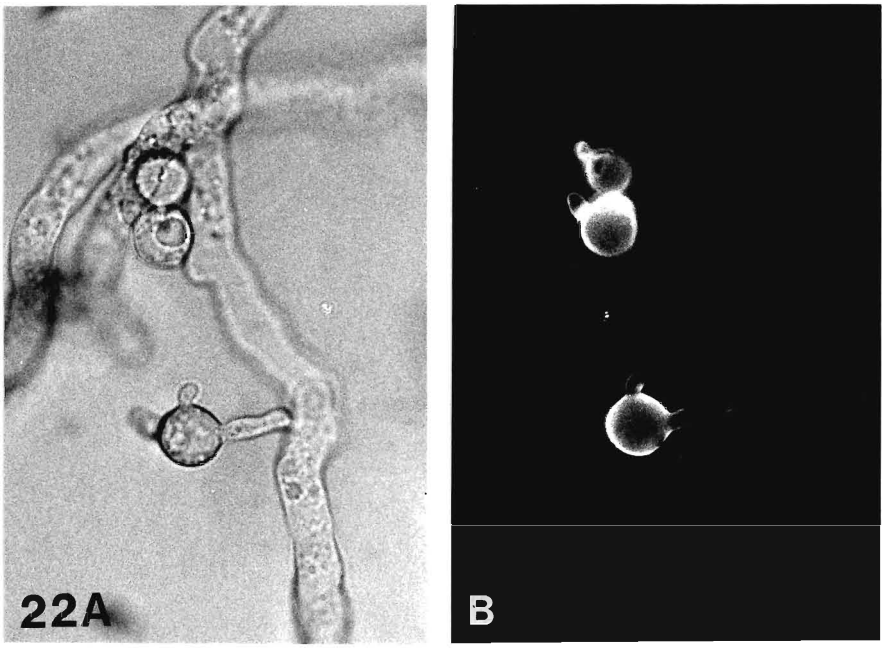
Part B. Chitinase Immuno-fluorescence and FITC-WGA Binding Assay During Host-Parasite Interaction

Typical infection structures of the mycoparasite, *P. virginiana* were found on the susceptible and resistant host within 20-22 h on the surface of the agar plates overlayed with a dialysis membrane. The binding patterns for both chitinase localization and WGA were identical in the susceptible host, *C. cucurbitarum* challenged by *P. virginiana* (Figs. 21A & B & 22A & B, respectively). Fluorescence was found along the entire cell surface of the mycoparasite but was not detected at all along the surface of *C. cucurbitarum.* This matched the results obtained with axenically grown *C. cucurbitarum* and *P. virginiana* (cf. Table1 and Part A of the results).

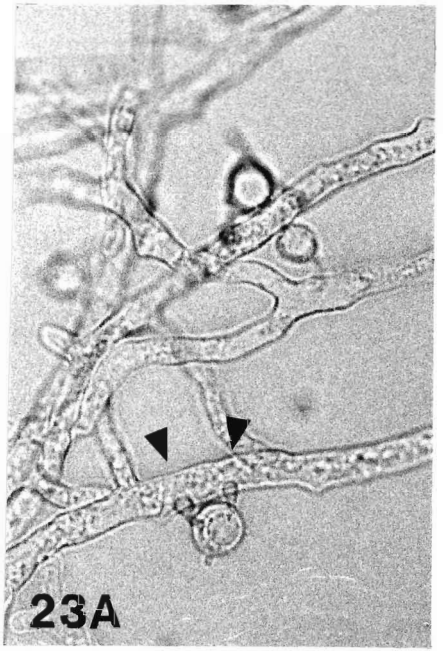
In case of the resistant host, strong fluorescence was detected using both probes along the cell surface of *P. articulosus* as well as the mycoparasite (Figs. 23 (A & B) & 24 (A & B); data represented is for FITC-WGA binding only). Appressoria formed on the surface of either host fluoresced with both probes as is clearly represented in Figures 23 (A & B; arrows) and 24 (A & B; arrows). Swollen spores of the mycoparasite attached to the cell surface of both hosts. Again, strong fluorescence was observed along the entire spore perimeter of *P. virginiana* with both probes (data not shown).

Figs. 21 & 22. Bright field and corresponding fluorescence photomicrographs of the susceptible host, *C. cucurbitarum* challenged with the mycoparasite, *P. virginiana*, 20 h post-infection X 600. **Indirect chitinase immuno-fluorescence** is illustrated in Fig. 21 (A & B) while **direct FITC-WGA binding** is represented in Fig. 22 (A & B). Note absence of fluorescence along the surface of *C. cucurbitarum* with both probes.

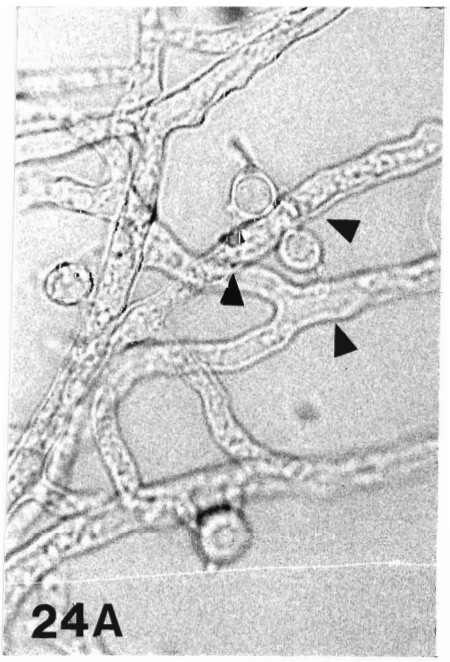

21A
B


22A
B

Figs. 23 & 24. Bright field and corresponding fluorescence photomicrographs of the resistant host, *P. articulosus* challenged with the mycoparasite, *P. virginiana*, 20 h post-infection X 600 with **direct FITC-WGA binding assay** only. Figures 23 (A & B) and 24 (A & B) differ only in depth of focus. Note prescence of strong fluorescence along the surface of both fungi and along the appressoria of the mycoparasite (arrows).


23A


B


24A


B

Discussion

The results of both immunoblot tests with the polyclonal antiserum towards the antigen from which it was derived, a microsomal chitinase isolated from *C. cucurbitarum,* suggests that this antiserum is indeed specific for chitinase. In the Western blot, one prominent and one faint band were seen in addition to a protein with a molecular mass of 66 kDa in the crude total cellular protein extract from *C. cucurbitarum.* Using SDS-PAGE, a purified chitinase isolated from the microsomal fraction of *C. cucurbitarum* migrated as a single, discrete band with a molecular mass of 66 kDa (Balasubramanian and Manocha, 1992). Therefore, this antiserum can appropriately be referred to as an anti-chitinase antiserum and is a suitable probe for immunocytochemical studies. The two other proteins may have similar antigenicity to the microsomal chitinase, hence the cross reactivity observed in the Western blot, but only the 66 kDa protein has chitinase activity (Balasubramanian and Manocha, 1992).

Cross reactivity of this antiserum was observed with two chitin binding lectins, WGA and PAA and plant ICF's from tobacco and barley. Prominent bands at M_r 27 kDa for tobacco and M_r 26 kDa for barley correspond to known molecular masses of acidic pathogenesis related proteins identified as chitinases in tobacco with molecular masses of 27.5, 28.5 kDa (Bol et al., 1990) and a 28 kDa chitinase identified in barley (Jacobsen et al., 1990), respectively. The presence of several bands in these extracts may represent isozymes of chitinases or other distinct proteins which share similar anitgenic epitopes. Cross reactivity was also found with lysozyme which is in agreement with recent evidence of lysozyme activity of chitinase preparations from *C. cucurbitarum* and *P. articulosus* (Balasubramanian and Manocha, 1992).

In this regard, it is known that many plant proteins share a 43 amino acid chitin binding domain as part of the protein structure (cf. pg. 33; Chrispeels and Raikhel, 1990). WGA, rice and barley lectins have four of these 43 amino acid long chitin binding domains. Class I plant chitinases are basic isoforms that contain two structural domains: a highly conserved cysteine and glycine-rich amino-terminal domain with chitin binding properties and a highly conserved chitinolytic catalytic domain, both interspersed with a short (8-11 amino acid) hyper-variable hinge region rich in glycine and proline residues (Shinshi et al., 1990; Neuhaus et al., 1991b). Basic isoforms of bean, tobacco, tomato, potato and an acidic bean chitinase fall into this category (Lawton et al., 1992).

Derived amino acid sequences from recently cloned fungal chitinase genes isolated from *Saccharomyces cerevisiae* and *Rhizopus oligosporus* also have one putative chitin binding domain (Kuranda and Robbins, 1991; Yanai et al., 1992). Moreover, a high extent of amino acid sequence homology of the chitinase catalytic domains from both these fungi has been shown towards catalytic domains present in plant chitinases and other ß-1,4 glycosidases (Kuranda and Robbins, 1991; Yanai et al., 1992). To our knowledge, this would be the first indirect conformation of chitinase isolated from a filamentous fungus to show similar antigenicity with both chitin binding lectins and chitinases isolated from higher plants. The presence of certain common protein structures may suggest a high extent of sequence homology for this particular enzyme from diverse taxonomic groups. Only direct comparisons of amino acid sequences could verify this assumption and may explain the cross reactivity observed.

There is clear evidence to suggest the direct involvement of chitinase in the processes of spore swelling, germination and sporangium development as shown by immuno-fluorescence microscopy. The uptake of water during spore swelling and emergence of the germ tube during germination of a spore would involve cell wall softening events which would dictate the need for a lytic enzyme. Cell wall softening during sporangium development and liberation of spores from the sporangium also would logically involve chitinolytic activity.

Indirect evidence involving chitinase during spore swelling and germination has been shown before. High chitinase activity in cytosolic extracts of mycelial form of *M. rouxii* has been correlated with the onset of germination in 4 h cultures (Pedraza-Reyes and Lopez-Romero, 1989). Recently, the same group described the identification of 9 chitinase species associated with a 4 h peak of chitinase activity that is expressed in swollen germ spheres just prior to the appearance of the germ tubes (Pedraza-Reyes and Lopez-Romero, 1991). In virulent isolates of the entomopathogen, *Nomuraea rileyi*, the chitinase activity/total protein ratio during germination (2 days) was as much as 35 times greater than that found in conidia at day 0 (El-Sayed et al., 1989). Iten and Matile (1970) correlated gross autolysis associated with stipe elongation and spore dispersal in *Coprinus lagopus* with the action of an endocellular chitinase synthesized shortly after autolysis began in the gills.

Perhaps most notable was the lack of chitinase localization at the hyphal apex and during new branch site formation with the techniques employed in this

study. Several lines of experimental evidence corroborate this result. Chitinase was not detected at the apex of any of the five fungi during germination or at later stages of growth. Mild proteolysis with commercial proteinases was attempted prior to labelling since it is known that chitinase exists in a zymogenic form activated by partial proteolytic degradation (Balasubramanian and Manocha, 1987). This failed to reveal chitinase at the apex or at newly emerging branch sites. Permeabilization of the cell surface prior to labelling also failed to reveal chitinase at the apex. Moreover, the same hyphae that were injured mechanically did not show any chitinase at the apex.

It could be possible that chitinase may be present in very small quantitites due to low enzyme turnover, be present in an inactive state or remain inaccessible to the antiserum for the lack of detection with the techniques employed in this study. Preliminary results from this study seem to favor the arguments of Wessels (1986) who supports the contention that autolysins like chitinase are not involved in apical hyphal growth.

There is clear evidence for the role of chitin synthetase during apical hyphal growth in zygomycetous fungi. Using autoradiography, incorporation of the radioactive precursor of chitin ([^{3}H]GlcNAc) was clearly observed at the apex of *C. cucurbitarum*. (Manocha and McCullough, 1985). Label incorporation was also found in mechanically injured hyphae of *C. cucurbitarum* within 5 min after sonication. Thus, new chitin microfibrils are being deposited at the apex and sites of physical injury by chitin synthetase. In the present study, chitinase was also detected at sites of mechanical injury and during germination of *C. cucurbitarum* when splitting of the sporangiospore cell wall took place. These results might suggest that chitinase is active at rupture sites and may be associated with chitin synthetase during physical injury.

Oligomers of GlcNAc or chitin microfibrils were detected at the apex of several fungi through a direct FITC-WGA binding assay. Indeed, the only difference between the binding patterns of chitinase localization with that of WGA was the fluorescence at the apex at various stages of growth. Such a result has been confirmed in various filamentous fungi (Mirelman et al., 1975; Manocha, 1985). Chemical analyses of isolated cell walls of *C. cucurbitarum, M. candelabrum, M. pusilla* and *P. articulosus* have shown that chitin is a major polysaccharide cell wall constituent (Manocha, 1981; Manocha, 1984). Differences in the dimensional arrangement of chitin microfibrils and the thickness of cell wall layers were also demonstrated. This apparent

contradiction can be explained by the fact that WGA will detect oligomers of GlcNAc only along the surface of the outermost cell wall layer. That chitin was present along the entire cell surface of some fungi while absent in others further illustrates the complexities of cell wall polysaccharide biochemistry even in so closely related fungi. Differences in cell surface binding patterns observed with WGA cannot be correlated with other host cell wall characteristics which may account for the extent and nature of parasitism by the mycoparasite, *P. virginiana* towards susceptible and resistant hosts and nonhosts. Although very similar in the patterns of binding, it is suggested that the lectin WGA should not be used as an indirect probe for the localization of chitinase as reported previously (Cherif and Benhamou, 1990).

Except for the apparent absence of chitinase and distinct presence of oligomers of GlcNAc during hyphal apical growth, the patterns of fluorescence obtained with both probes were identical during typical biotrophic mycoparasite interactions. Several experiments have been performed in this laboratory in order to measure both response during penetration and properties of chitin synthestase activity *in vitro*, in susceptible and resistant hosts challenged by the mycoparasite, *P. virginiana* (Manocha and Graham, 1982; Manocha and Begum, 1985). Characterization of chitin synthetase response, both *in vivo* and *in vitro*, and morphological observations during infection has led to a hypothesis proposing higher levels of chitinase activity in the susceptible host and chitin synthestase activity in the resistant host when challenged by the mycoparasite (see Manocha, 1987 for details). Manocha and McCullough (1985) demonstrated localized incorporation of [^{3}H]GlcNAc at the penetration sites on the resistant but not the susceptible host. However, no work has been done on the response of chitinase in both hosts during early stages of host-mycoparasite interaction. Since the patterns of fluorescence with the anti-chitinase probe were the same in each fungus during early stages of mycoparasitism as if they were grown axenically, results from this part of the study cannot prove or disprove the hypothesis proposed by Manocha (1987). Experimental support for this hypothesis due to the extent and distribution of chitinase and/or chitin based only on immunocytochemical techniques may be adequately obtained at the electron microscope level using colloidal gold labelled probes.

Few investigators have attempted to clone chitinase genes in filamentous fungi as compared to numerous such reports for plant chitinases. These studies have provided circumstantial evidence for the role of plant chitinases during

morphogenesis in transgenic plants, elucidated derived structural domains from cDNA clones of different chitinase classes and provided speculation for the origin of plant chitinases. Thus, future studies must utilize colloidal-gold immuno-cytochemical techniques for chitinase and chitin due to greater spatial localization and molecular methods to clone chitinase genes in order to clarify the role of fungal chitinases in morphogenesis and host-parasite interactions.

Conclusions

1. A polyclonal anti-chitinase antiserum is specific for its antigen, microsomal chitinase isolated from the fungus, *Choanephora cucurbitarum,* and showed cross reactivity with other fungal chitinases and bacterial chitinases and chitin-binding lectins from plant sources.

2. Chitinase seems to be directly involved in the processes of spore swelling, germination, sporangium formation and response to mechanical injury and could not be detected during hyphal apical growth or during new branch site initiation.

3. Patterns of chitinase localization directly match the patterns of localization of oligomers of GlcNAc at various developmental stages of axenic growth and during typical host-biotrophic mycoparasite interactions, except at the apex and during new branch site initiation where only oligomers of GlcNAc are localized.

4. Although very similar, the two chitin-binding probes, chitinase and WGA differ in localization patterns and it is not encouraged that WGA be used as an indirect probe for chitinase localization.

References

Abeles, F.B., Bosshart, R.P., Forrence, L.E. and Hibig, W.H. 1971. Preparation and purification of glucanase and chitinase from bean leaves. Plant Physiol. **47:** 129-134.

Audy, P., le Quere, D., Leclerc, D. and Asselin, A. 1990. Electrophoretic forms of lysozyme activity in various plant species. Phytochemistry **29:** 1143-1159.

Bade, M. L. and Hickey, K. 1989. Classification of enzymes hydrolyzing chitin. *In* Chitin and Chitosan, sources, chemistry, biochemistry, physical properties and applications *Edited by* G. Skjak-Bræk, T. Anthonsen, and P. Sandford. Elsevier, London. pp. 179-184.

Balasubramanian, R. and Manocha, M.S. 1986. Proteinase, chitinase, and chitosanase activities in germinating spores of *Piptocephalis virginiana.* Mycologia **78:** 157-163.

Balasubramanian, R. and Manocha, M.S. 1992. Cytosolic and membrane bound chitinases of two mucoraceous fungi: a comparitive study. Can. J. Microbiol. **38:** 331-338.

Barrett-Bee, K. and Hamilton, M. 1984. The detection and analysis of chitinase activity from the yeast form of *Candida albicans.* J. Gen. Microbiol. **130:** 1857-1861.

Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu. Rev. Microbiol. **22:** 87-108.

Bartnicki-Garcia, S. 1973. Fundamental aspects of hyphal morphogenesis. *In* Microbial differentiation *Edited by* J.M. Ashworth and J.E. Smith. Cambridge University Press, London. pp. 245-267.

Blaiseau, P.L., Kunz, C., Grison, R., Bertheau, Y. and Brygoo, Y. 1992. Cloning and expression of a chitinase gene from the hyperparasitic fungus *Aphanocladium album.* Curr. Genet. **21:** 61-66.

Bol, J.F., Linthorst, H.J.M. and Cornelissen, B.J.C. 1990. Plant pathogenesis-related proteins induced by virus infection. Annu. Rev. Phytopathol. **28:** 113-138.

Boller, T. 1987 Hydrolytic enzymes in plant disease resistance *In* Plant-microbe interactions, molecular and genetic perspectives. Vol. 2. *Edited by* T. Kosuge and E.W. Nester. MacMillan, New York. pp. 384-414.

Boller, T., Gehri, A., Mauch, F. and Vogeli, U. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties and possible function. Planta **157:** 22-31.

Boller, T. and Vogeli, U. 1984. Vacuolar localization of ethylene-induced chitinase in bean leaves. Plant Physiol. **74:** 442-444.

Bowles, D.J. 1990. Defense-related proteins in higher plants. Annu. Rev. Biochem. **59:** 73-907.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. **72:** 248-254.

Bradshaw, H.D. 1990. Killer toxins. Nature **345:** 299.

Broekaert, W.F. and Peumans, W.J. 1988. Pectic polysaccharides elicit chitinase accumulation in tobacco. Physiologia Plantarum **74:** 740-744.

Broekaert, W.F., Parijs, J.V., Leyns, F., Joos, H. and Peumans, W.J. 1989. A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. Science **245:** 1100-1102.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C. J. and Broglie, R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani.* Science **254:** 1194-1197.

Butler, A.R., O'Donnell, R.W., Martin, V.J., Gooday, G.W. and Starck, M.J.R. 1991. *Kluveromyces-lactis* toxin has essential chitinase activity. Eur. J. Biochem. **199:** 483-488.

Cabib, E. 1987. The synthesis and degradation of chitin. Adv. Enzymol. **59:** 59-101.

Cabib, E., Silverman, S.J. and Shaw, J.A. 1992. Chitinase and chitin synthase 1: counterbalancing activities in cell separation of *Saccharomyces cerevisiae.* J. Gen. Microbiol. **138:** 97-102.

Cherif, M. and Benhamou, N. 1990. Cytochemical aspects of chitin breakdown during the parasitic action of a *Trichoderma* sp. on *Fusarium oxysporum* f. sp. *radicis-lycopersici.* Phythopathol. **80:** 1406-1414.

Chrispeels, M.J. and Raikhel, N.V. 1991. Lectins, lectin genes, and their role in plant defense. Plant Cell **3:** 1-9.

Correa, J.U., Elango, N., Polacheck, I. and Cabib, E. 1982. Endochitinase, a mannan associated enzyme from *Saccharomyces cerevisiae.* J. Biol. Chem. **257:** 1392-1397.

Coudron, T.A., Kroha, M.J., Ignoffo, C.M. 1984. Levels of chitinolytic activity during development of three entomopathogenic fungi. Comp. Biochem. Physiol. **79B:** 339-348.

De La Cruz, J., Hidalgogallego, A., Lora, J.M., Benitez, T. and Pintortoro, J.A. and Leobell, A. 1992. Isolation and characterization of 3-chitinases from *Trichoderma harzianum.* Eur. J. Biochem **206:** 859-867.

de Jong, A.J., Cordewener, J., Lo Sciavo, F., Terzi, M., Vandekerckhove, J., Van Kammen, A. and de Vries, S. 1992. A carrot somatic embryo mutant is rescued by chitinase. Plant Cell **4:** 425-433.

del Campillo, E. and Lewis, L.N. 1992. Occurrence of 9.5 cellulase and other hydrolases in flower reproductive organs undergoing major cell wall disruption. Plant Physiol. **99:** 1015-1020.

Dickinson, K., Keer, V., Hitchcock, C.A. and Adams, D.J. 1989.Chitinase activity from *Candida albicans* and its inhibition by allosamidin. J. Gen. Microbiol. **135:** 1417-1421.

Dickinson, K., Keer, V., Hitchcock, C.A. and Adams, D.J. 1991. Microsomal chitinase activity from *Candida albicans.* Biochim. Biophys. Acta **1073:** 177-182.

Di Pietro, A., Lorito, M., Hayes, C.K., Broadway, R.M. and Harman, G.E. 1993. Endochitinase from *Gliocladium virens:* isolation, characterization, and synergistic antifungal activity in combination with gliotoxin. Phytopathol. **83:** 308-313.

Dumas-Gaudot, E., Grenier, J., Furlan, V. and Asselin, A. 1992. Chitinase, chitosanase and ß-1,3 glucanase activities in *Allium* and *Pisum* roots colonized by *Glomus* species. Plant Science **84:** 17-24.

El-Sayed, G.N., Coudron, T.A. and Ignoffo, C.M. 1989. Chitinolytic activity and virulence associated with native and mutant isolates of the entomopathogenic fungus, *Nomuraea rileyi.* J. Inv. Pathol. **54:** 394-403.

Elad, Y., Barak, R. and Chet, I. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum.* Can. J. Microbiol. **28:** 719-725.

Elango, N., Correa, J.U. and Cabib, E. 1982. Secretory character of yeast chitinase. J. Biol. Chem. **257:** 1398-1400.

Esaka, M., Enoki, K., Kouchi, B. and Sasaki, T. 1990. Purification and characterization of abundant secreted protein in suspension-cultured pumpkin cells. Plant Physiol. **93:** 1037-1041.

Gooday, G. 1990. The ecology of chitin degradation. Microbial Ecol. **10:** 387-431.

Garcia Breijo, F.J., Garro, R. and Conejero, V. 1990. C7(P32) and C6(P34) PR proteins induced in tomato leaves by citrus exocortis viroid infection are chitinases. Physiol. Mol. Plant Pathol. **36:** 249-260.

Grenier, J., and Asselin, A. 1990. Some pathogenesis-related proteins are chitosanases with lytic activity against fungal spores. Mol. Plant-Microbe Int. **3:** 401-407.

Grosset, J., Meyer, Y., Chartier, Y., Kauffman, S., Legrand, M. and Fritig, B. 1990. Tobacco mesophyll protoplasts synthesize 1,3-ß-glucanase, chitinases, and "osmotins" during *in vitro* culture. Plant Physiol. **92:** 520-527.

Gupta, S.C., Leathers, T.D., El-Sayed, G.N. and Ignoffo, C.M. 1991. Production of degradative enzymes by *Metarhizium anisopliae* during growth on defined media and insect cuticle. Exp. Mycol. **15:** 310-315.

Ham, K.-S., Kauffmann, S., Albersheim, P., and Darvill, A.G. 1991. Host-pathogen interactions XXXIX. A soybean pathogenesis-related protein with ß-1,3-glucanase activity releases phytoalexin elicitor-active heat-stable fragments from fungal walls. Mol. Plant-Microbe Interact. **4:** 545-552.

Harikrishna, K., Jampates-Beal, R. and Charles, S. 1991. A basic chitinase is expressed at high levels in the transmitting tissues of tomatoes. *In* Molecular biology of plant growth and development, program and abstracts *Edited by* R.B. Hallick. Department of Biochemistry, University of Arizona, Tuscon. abstr. 511.

Harman, G.E., Hayes, C.K., Lorito, M., Broadway, R.M., Di Pietro, A., Peterbauer, C. and Tronsmo, A. 1993. Chitinolytic enzymes of *Trichoderma harzianum:* purification of chitobiosidase and endochitinase. Phytopathol. **83:** 313-318.

Harris, J.L. and Szaniszlo, P.J. 1986. Localization of chitin in walls of *Wangiella dermatidis.* Mycologia **78:** 853-857.

Heath, I.B. 1990. The roles of actin in tip growyh of fungi. Int. Rev. Cytol.**123:** 95-127.

Hedrick, S.A., Bell, J.N., Boller, T. and Lamb, C.J. 1988. Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding, and infection. Plant Physiol. **86:** 182-186.

Humphreys, A.M. and Gooday, G.W. 1984. Properties of chitinase activities from *Mucor mucedo:* evidence for membrane-bound zymogenic form. J. Gen. Microbiol. **130:** 1359-1366.

Hurn, B.A.L., and Chantler, S.M. 1980. Production of reagent antibodies. Meth. Enzymol. **70:** 104-142.

Huynh, Q.K., Hironaka, C.M., Levine, E.B., Smith, C.E., Borgmeyer, J.R. and Shah, D.M. 1992. Antifungal proteins from plants-purification, molecular cloning, and antifungal properties of chitinases from maize seeds. J. Biol. Chem. **267:** 6635-6640.

Issac, S. and Gokhale, A.V. 1982. Autolysis: a tool for protoplast production from *Aspergillus nidulans.* Trans. Br. Mycol. Soc. **78:** 389-394.

Iten, W. and Matile, P. 1970. Role of chitinase and other lysosomal enzymes of *Coprinus lagopus* in the autolysis of fruiting bodies. J. Gen. Microbiol. **61:** 301-309.

Jackson, C.W., Heale, J.B. and Hall, R. A. 1985. Traits associated with virulence to the aphid *Macrosiphoniella sanborni* to eighteen isolates of *Verticillium lecanii.* Ann. Appl. Biol. **106:** 39-48.

Jacobsen, S., Mikkelsen, J.D. and Hejgaard, J. 1990. Characterization of two antifungal endochitinases from barley grain. Physiologia Plantarum **79:** 554-562.

Jacobsen, S., Hauschild, M.Z. and Rasmussen, U. 1992. Induction of chromium ions of chitinases and polyamines in barley (Hordeum-vulgaris L) and rape (*Brassica-napus* L S.sp. *oleifera*). Plant Science **84:** 119-128.

Kaminskyj, S.G.W., Garrill, A., and Heath, I.B. 1992. The relation between turgor and tip growth in *Saprolegnia ferax:* turgor is necessary, but not sufficient to explain apical extension rates. Exp. Mycol. **16:** 64-75.

Keen, N.T., Yoshikawa, M. and Wang, M.C. 1983. Phytoalexin elicitor activity of carbohydrates from *Phytophthora megasperma* f. sp. *glycenia* and other sources. Plant Physiol. **71:** 466-471.

Knowles, J., Lehtovaara, P. and Tuula, T. 1987. Cellulase families and their genes. Trends Biotechnol. **5:** 255-261.

Ko, W-.H. and Lockwood, J.L. 1970. Mechanism of lysis of fungal mycelia in soil. Phytopathol. **60:** 148-154.

Kombrink, E., Schroder, M. and Hahlbrock, K. 1988. Several "pathogenesis-related" proteins in potato are 1,3-ß-glucanases and chitinases. Proc. Nat'l. Acad. Sci. USA. **85:** 782-786.

Kragh, K.M., Jacobsen, S., Mikkelsen, J.D. and Nielsen, K.A. 1991. Purification and characterization of three chitinases and one ß-1,3-glucanase accumulating in the medium of cell suspension cultures of barley (*Hordeum vulgare* L.). Plant Science **76:** 65-77.

Kunz, C., Sellam, O. and Bertheau ,Y. 1992. Purification and characterization of a chitinase from the hyperparastic fungus *Aphanocladium album.* Physiol. Mol. Plant Pathol. **40:** 117-131.

Kuranda, M.J. and Robbins, P.W. 1991. Chitinase is required for cell separation during the growth of *Saccharomyces cerevisiae.* J. Biol. Chem. **266:** 19758-19767.

Kurosaki, F., Tashiro, N. and Nishi, A. 1987. Secretion of chitinase from cultured carrot cells treated with fungal mycelial walls. Physiol. Mol. Plant Pathol. **31:** 211-216.

Lambais, M.R. and Mehdy, M.C. 1993. Suppression of endochitinase, ß-1,3-endoglucanase, and chalcone isomerase expression in bean vesicular-arbuscular mycorrhizal roots under different soil phosphate conditions. Mol. Plant-Microbe Int. **6:** 75-83.

Lawton, K., Ward, E., Payne, G., Moyer, M. and Ryals, J. 1992. Acidic and basic class-III chitinase messenger RNA accumulation in response to TMV infection of tobacco. Plant Mol. Biol. **19:** 735-743.

Lerner, D.R. and Raikhel, N. 1992. The gene for stinging nettle lectin (*Urtica dioica* agglutinin) encodes both a lectin and a chitinase. J. Biol. Chem. **267:** 11085-11091.

Leung, D.W.M. 1992. Involvement of plant chitinase in sexual reproduction of higher plants. Phytochemistry **31:** 1899-1900.

Lopez-Romero, E., Ruiz-Herrera, J. and Bartnicki-Garcia, S. 1978. Purification and properties of an inhibitory protein of chitin synthetase from *Mucor rouxii.* Biochim. Biophys. Acta **525:** 338-345.

Lopez-Romero, E., Ruiz-Herrera, J. and Bartnicki-Garcia, S. 1982. The inhibitory protein of chitin synthetase from *Mucor rouxii* is a chitinase. Biochim. Biophys. Acta **702:** 233-236.

Lorito, M., Harman, G.E., Hayes, C.K., Broadway, R.M., Tronsmo, A., Woo, S.L. and Di Pietro, A. 1993. Chitinolytic enzymes produced by *Trichoderma harzianum:* antifungal activity of purified endochitinase and chitobiosidase. Phytopathol. **83:** 302-307.

Manocha, M.S. 1981. Host specificity and mechanism of resistance in a mycoparasite system. Physiol. Plant Pathol. **18:** 257-265.

Manocha, M.S. 1984. Cell surface characteristics of *Mortierella* species and their interaction with a mycoparasite. Can. J. Microbiol. **30:**290-298.

Manocha, M.S. 1985. Specificity of mycoparasite attachment to the host cell surface. Can. J. Bot. **63:** 772-778.

Manocha, M.S. 1987. Cellular and molecular aspects of fungal host-mycoparasite interaction. J. Pl. Dis. Prot. **94:** 431-444.

Manocha, M.S. and Balasubramanian, R. 1988. *In vitro* regulation of chitinase and chitin synthetase activity of two mucoraceous hosts of a mycoparasite. Can. J. Microbiol. **34:** 1116-1121.

Manocha, M.S. and Begum, A. 1985. Properties of chitin synthetase from mucoraceous hosts of a mycoparasite. Can. J. Microbiol. **31:** 6-12.

Manocha, M.S. and Golesorkhi, R. 1981. Host-parasite relations in a mycoparasite. VII. light and scanning electron microscopy of interactions of *Piptocephalis virginiana* with host and non-host species. Mycologia **73:** 976-987.

Manocha, M.S., and McCullogh, C.M. 1985. Suppression of host cell wall synthesis at penetration sites in a compatible interaction with a mycoparasite. Can. J. Bot. **63:** 967-973.

Manocha, M.S., and Sahai, A.S. 1991. Cell surface characterization of five haustorial mycoparasites with lectin probes. Mycologia **83:** 643-649.

Mauch, F. and Staehelin, L.A. 1989. Functional implications of the subcellular localization of ethylene-induced chitinase and ß-1,3-glucanase in bean leaves. Plant Cell **1:** 447-457.

Mauch, F., Hadwiger, L.A. and Boller, T. 1984. Ethylene: symptom, not signal for the induction of chitinase and ß-1,3-glucanase in pea pods by pathogens and elicitors. Plant Physiol. **76:** 607-611.

Mauch, F., Hadwiger, L.A. and Boller, T. 1988a. Antifungal hydrolases in pea tissue. I. Purification and characterization of two chitinases and two ß -1,3 glucanases differentially regulated during development in response to fungal infection. Plant Physiol. **87:** 325-333.

Mauch, F., Mauch-Mani, B. and Boller, T. 1988b. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and ß-1,3 glucanase. Plant Physiol. **88:** 936-942.

McMurrough, I. and Bartnicki-Garcia, S. 1973. Inhibition and activation of chitin synthesis by *Mucor rouxii* cell extracts. Arch. Biochem. Biophys. **158:** 812-816.

Metraux, J.P. and Boller, T. 1986. Local and systemic induction of chitinase of cucumber plants in response to viral, bacterial and fungal infections. Physiol. Mol. Plant Pathol. **28:** 161-169.

Milewski, S., O'Donnell, R.W. and Gooday, G.W. 1992. Chemical modification studies of the active centre of *Candida albicans* chitinase and its inhibition by allsoamidin. J. Gen. Microbiol. **138:** 2545-2550.

Mirelman, D., Galun, E., Sharon, N. and Lotan, R. 1975. Inhibition of fungal growth by wheat germ agglutinin. Nature **256:** 414-416.

Molano, J., Polachek, I., Duran, A. and Cabib, E. 1979. An endochitinase from wheat germ: activity on nascent and preformed chitin. J. Biol. Chem. **254:** 4901-4907.

Nasser, W., deTapia, M. and Burkard, G. 1990. Maize pathogenesis-related proteins characterization and cellular distribution of 1,3-ß-glucanases and chitinases induced by brome mosaic virus infection or mercuric chloride treatment. Physiol. Mol. Plant Pathol. **36:** 1-14.

Neuhaus, J.M., Ahi-Goy, P., Hinz, U., Flores, S. and Meins, F., Jr. 1991a. High level expression of a tobacco chitinase gene in *Nicotiana silvestris:* susceptibility of transgenic plants to *Cercospora nicotianae.* Plant Mol. Biol. **16:** 141-151.

Neuhaus, J.M., Sticher, L., Meins, F., Jr. and Boller, T. 1991b. A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc. Nat'l. Acad. Sci. USA. **88:** 10362-10366.

Ohashi, Y. and Matsuoka, M. 1985. Synthesis of stress proteins in tobacco leaves. Plant Cell Physiol. **26:** 473-480.

Ohashi, Y. and Matsuoka, M. 1987. Induction and secretion of pathogenesis related proteins by salicylate or plant hormones in tobacco suspension cultures. Plant Cell Physiol. **28:** 573-580.

Ordentlich, A., Migheli, Q. and Chet, I. 1991. Biological control activity of three *Trichoderma* isolates against *Fusarium* wilts of cotton and muskmelon and lack of correlation with their lytic enzymes. J. Phytopathol. **133:** 177-186.

Pan, S. Q., Ye, X.S.and Kuc, J. 1992. Induction of chitinases in tobacco plants systemically protected against blue mold by *Peronospora tabacina* or tobacco mosaic virus. Phytopathol. **82:** 119-123.

Parent, J. G., Belanger, F., Desjarrdin, S., and Brisson, J. D. 1985. Dot-immunobinding for the detection of tomato mosaic virus and potato virus in infection greenhouse tomato. Phytoprotection. **66:** 53-57.

Pedraza-Reyes, M. and Lopez-Romero, E. 1989. Purification and some properties of two forms of chitinase from mycelial cells of *Mucor rouxii.* J. Gen. Microbiol. **135:** 211-218.

Pedraza-Reyes, M. and Lopez-Romero, E. 1991. Detection of nine chitinase species in the germinating cells of *Mucor rouxii.* Curr. Microbiol. **22:** 43-46.

Pegg, G.F. 1988 Chitinase from *Verticillium albo-atrum.* Meth. Enzymol. **161:** 474-479.

Pegg, G.F. and Young, D.H. 1981. Changes in glycosidase activity and their relationship to fungal colonization during infection of tomato by *Verticillium albo-atrum.* Physiol. Plant Pathol. **19:** 371-382.

Penefsky, H. S., and Tzagoloff, A. 1971. Extraction of water-soluble enzymes and proteins from membranes. Meth. Enzymol. **22:** 204-219.

Phipps, P. M., and Barnett, H. L. 1975. Effect of nitrogen nutrition on the free amino acid pool of *Choanephora cucurbitarum* and parasitism by two haustorial mycoparasites. Mycologia **67:** 1128-1142.

Polacheck, I. and Rosenberger, R.F. 1978. Distribution of autolysins in hyphae of *Aspergillus nidulans:* evidence for a lipid mediated attachment to hyphal cell walls. J. Bact. **135:** 741-747.

Rast, D.M., Horsch, M., Furter, R. and Gooday, G. 1991. A complex chitinolytic system in exponentially growing mycelium of *Mucor rouxii:* properties and function. J. Gen. Microbiol. **137:** 2797-2810.

Reyes, F., Calatayud, J. and Martinez, M.J. 1989. Endochitinase from *Aspergillus nidulans* implicated in the autolysis of its cell wall. FEMS Microbiol. Lett. **60:** 119-124.

Robbins, P.W., Albright, C. and Benfield, B. 1988. Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Eschericia coli.* J. Biol Chem. **263:** 443-447.

Roberson, R. W. 1992. The actin cytoskeleton in hyphal cells of *Scelortium rolfsii.* Mycologia **84:** 41-51.

Roberts, W.K. and Selitrennikoff, C. L. 1988. Plant and bacterial chitinases differ in anti-fungal activity. J. Gen. Microbiol. **134:** 169-176.

Roby, D., Toppan, A., Esquerre-Tugaye, M. 1986. Cell surfaces in plant-microorganism interactions. VI. Elicitors of ethylene from *Colletotrichum lagenarium* trigger chitinase. Plant Physiol. **81:** 228-233.

Rozeboom, H.J., Budiani, A., Beintema, J.J. and Dijkstra, B.W. 1990. Crystallization of hevamine, an enzyme with lysozyme/chitinase activity from *Hevea brasiliensis* latex. J. Mol. Biol. **212:** 441-443.

Samac, D.A., and Shah, D.M. 1991. Developmental and pathogen-induced activation of the *Arabidopsis* acidic chitinase promoter. Plant Cell **3:** 1063-1072.

Schulbaum, A., Mauch, F., Vogeli, U. and Boller, T. 1986. Plant chitinases are potent inhibitors of fungal growth. Nature **324:** 365-367.

Shapira, R., Ordentlich, A., Chet, I. and Oppenheim, A.B. 1989. Control of plant diseases by chitinase expressed from cloned DNA in *Eschericia coli.* Phytopathol. **79:** 1246-1249.

Shinshi, H., Mohnen, D. and Meins Jr., F. 1987. Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitinase mRNA accumulation in cultures tobacco tissues by auxin and cytokinin. Proc. Nat'l. Acad. Sci. USA **84:** 89-93.

Shinshi, H., Neuhaus, J.M., Ryals, J. and Meins Jr., F. 1990. Structure of a tobacco endochitinase gene: evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. Plant Mol. Biol. **14:** 357-368.

Sivan, A. and Chet, I. 1989. Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum.* J. Gen. Microbiol. **135:** 675-682.

Smith, J. and Grula, E.A. 1983. Chitinase is an inducible enzyme in *Beauveria bassiana.* J. Inv. Pathol. **42:** 319-326.

Spanu, P., Boller, T., Ludwig, A., Wiemken, A., Faccio, A. and Bonfante-Fasolo, P. 1989. Chitinase in roots of mycorrhizal *Allium porrum:* regulation and localization. Planta **177:** 447-455.

Srivastava, A.K., Defago, G. and Boller, T. 1985. Secretion of chitinase by *Aphanocladium album,* a hyperparasite of wheat rust. Experientia **41:** 1612-1613.

St. Leger, R., Cooper, R.M. and Charnley, A.K. 1986. Cuticle degrading enzymes of entomopathogenic fungi: regulation of production of chitinolytic enzymes. J. Gen. Microbiol. **132:** 1509-1517.

St. Leger, R., Cooper, R.M., and Charnley, A.K. 1987. Production of cuticle-degrading enzymes by the entomopathogen *Metarhizium anisopliae* during infection of cuticles from *Calliphora vomitaria* and *Manduca sexta.* J. Gen. Microbiol. **133:** 1371-1382.

St. Leger, R., Cooper, R.M. and Charnley, A.K. 1991. Characterization of chitinase and chitobiase produced by the entomopathogenic fungus *Metarhizium anisopliae.* J. Inv. Pathol. **58:** 415-426.

Sticher, L., Hofsteenge, J., Milani, A., Neuhaus, J.M. and Meins, F-Jr. 1992. Vacuolar chitinases of tobacco: a new class of hydroxyproline-containing proteins. Science **257:** 655-657.

Stirling, J.L., Cook, G.A. and Pope A.M.S. 1979. Chitin and it degradation. *In* Fungal walls and hyphal growth *Edited by* J.H. Burnett and A.P.S. Trinci. Cambridge University Press, New York. pp. 169-188.

Swegle, M., Kramer, K.J. and Muthukrishnan, S. 1992. Properties of barley seed chitinases and release of embryo-associated isoforms during early stages of imbibition. Plant Physiol. **99:** 1009-1014.

Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedures and some applications Proc. Nat'l. Acad. Sci. USA **76:** 4350-4354.

Ulhoa, C.J. and Peberdy, J.F. 1991. Regulation of chitinase synthesis in *Trichoderma harzianum.* J. Gen. Microbiol. **137:** 2163-2169.

Ulhoa, C.J. and Peberdy, J.F. 1992. Purification and some properties of the extracellular chitinase produced by *Trichoderma harzianum.* Enzyme Microb. Technol. **14:** 236-240.

Ulhoa, C.J. and Peberdy, J.F. 1993. Effect of carbon sources on chitobiase production by *Trichoderma harzianum.* Mycol. Res. **97:** 45-48.

Van Parijs, J., Broekaert, W.F., Goldstein, I.J., Peumans, W.J. 1991. Hevein: an antifungal protein from rubber-tree (Hevea brasiliensis) latex. Planta **183:** 258-264.

Vasseur, V., Arigoni, F., Andersen, H., Defago, G., Bompeix, G. and Seng, J. 1990. Isolation and characterization of *Aphanocladium album* chitinase-overproducing mutants. J. Gen. Microbiol. **136:** 2561-2567.

Verburg, J.G. and Huynh, Q.K. 1991. Purification and characterization of an antifungal chitinase from *Arabidopsis thailana.* Plant Physiol. **95:** 450-455.

Verburg, J.G., Smith, C.E., Lisek, C.A. and Huynh, Q.K. 1992. Identification of an essential tyrosine residue in the catalytic site of a chitinase isolated from *Zea mays* that is selectively modified during inactivation with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. J. Biol. Chem. **267:** 3886-3893.

Vessey, J.C. and Pegg, G.F. 1973. Autolysis and chitinase production in cultures of *Verticillium albo-atrum.* Trans. Br. Mycol. Soc. **60:** 133-143.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J-.P. and Ryals, J.A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3:** 1085-1094.

Wessels, J.G.H. 1986. Cell wall sythesis in apical hyphal growth. Int. Rev. Cytol. **104:** 37-79.

Yanai, K., Takaya, N., Kojima, N., Horiuchi, H., Ohta, A. and Takagi, M. 1992. Purification of two chitinases from *Rhizopus oligosporus* and isolation and sequencing of the encoding genes. J. Bact. **174:** 7398-7406.

Yoshikawa, M., Takeuchi, Y. and Horino, O. 1990. A mechanism for ethylene-induced disease resistance in soybean: enhanced synthesis of an elicitor-releasing factor, ß-1,3-endoglucanase. Physiol. Mol. Plant Pathol. **37:** 367-376.

Young, D.H. and Pegg, G.F. 1981. Purification and characterization of 1,3-ß-glucan hydrolases from healthy and *Verticillium albo-atrum*-infected tomato plants. Physiol. Plant Pathol. **19:** 391-417.

Young, D.H. and Pegg, G.F. 1982. The action of tomato and *Verticillium albo-atrum* glycosidases on the hyphal wall of *Verticillium albo-atrum.* Physiol. Plant Pathol. **21:** 411-423.

Yuan, S., and Heath, I. B., 1991. Chlorotetracycline staining patterns of growing hyphal tips of the oomycete *Saprolegnia ferax.* Exp. Mycol. **15:** 91-102.

Zarain-Herzberg, A. and Arroyo-Begovich, A. 1983. Chitinolytic activity from *Neurospora crassa.* J. Gen. Microbiol. **129:** 3319-3326.